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PREFACE

This volume of the *Annual Review of Plant Physiology* marks a decade of service to teaching and research in the field of plant physiology. The ten books reflect the efforts of a very considerable body of scholars.

In the beginning, the Board of Directors of Annual Reviews, Inc. and its managing editor, J. Murray Luck, agreed to sponsor the review series. The subject matter and authors for each volume have been selected by an Editorial Committee whose original membership consisted of D. R. Goddard, P. J. Kramer, A. E. Murneek, M. W. Parker, and K. V. Thimann. From time to time, in accordance with the rotation policy, the original members have been replaced; and, in 1957, the committee was enlarged to permit the inclusion of reviews on the physiology of plant disease. In chronological order the committee has included, in addition to the original members, H. B. Tukey, J. Bonner, C. A. Swanson, K. V. Thimann (second term), S. B. Hendricks, F. E. Gardner, J. G. Horsfall, and A. Lang. In addition, at each place of meeting, the committee has had one or more of the local plant physiologists as guest members. This has not only given a broader base to our deliberations but has made it possible for a number of colleagues to see from the inside the workings of an Annual Review.

C. A. Swanson completed his term of service on December 31, 1958. His excellent contributions are gratefully acknowledged. Many thanks. His successor is H. Beevers.

An editor of these volumes has several tasks. First, he must organize and then chair the annual meeting of the Editorial Committee. Then, after he has issued the invitations to prospective authors and received the desired affirmative replies, there is a quiet period of some fifteen months while the authors are preparing their reviews. During this period one or two may find that they cannot fulfill their obligation and have to be replaced, or the reviews must be postponed or dropped. The next eight months are fulfilling and busy ones. The manuscripts arrive (hopefully!) to be edited, as are later the galleys and then the page proofs. The editor and associate editor share equally in this work, and the editorial assistants in Palo Alto strive to keep up with their chores. For the first six years, D. I. Arnon was editor, assisted by the present writer. He was followed by L. R. Blinks, who functioned as editor for a year; then A. S. Crafts, for two years; and, finally, during the past year, the present incumbent. Since 1956, J. G. Torrey has been an associate editor. Not to be forgotten are the series of editorial assistants and those who prepared the indexes, even though they are not recorded here by name.

In preparing this brief tribute to all those who have made a tenth anniversary possible, I have reserved for the last those who have provided the meat and sinew of the volumes—the authors of the reviews. Altogether, 158 reviews and two prefatory chapters have been published. These were written

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by 207 different authors, of whom fifteen contributed to two reviews and two participated in three articles. Were these authors to be listed, their international representation and diversity of interests would be self-evident. At the time of preparing their reviews, they were resident in the following countries: Australia, Canada, Finland, France, Germany, Great Britain, Israel, Japan, Netherlands, Sweden, Switzerland, Turkey, U.S.A., U.S.S.R., and Venezuela.

Thus, somewhat more than 220 scholars altogether have participated in this enterprise. The endeavor is clearly a cooperative one. With the ever-increasing flow of research, the cooperation of an even larger number of scientists will be necessary for the *Annual Review of Plant Physiology* to continue its service effectively.

This tenth volume was planned by the Editorial Committee listed elsewhere and initiated under the editorship of L. R. Blinks. It incubated under the guidance of A. S. Crafts, and was prepared for publication by the present incumbent. Throughout this period, the associate editor, J. G. Torrey, performed service beyond the normal duties of his position. I also wish to acknowledge the contributions of the editorial assistants for Volume 10, Sonya Pinney, Rena Gerber, Florene Dick, Peggy Zimmers, and Anne Bogley, as well as those of Grace MacNeill who prepared the subject index. As usual, Dr. J. Murray Luck was ever ready with assistance and advice and the George Banta Company with its cooperation.

L. MACHLIS

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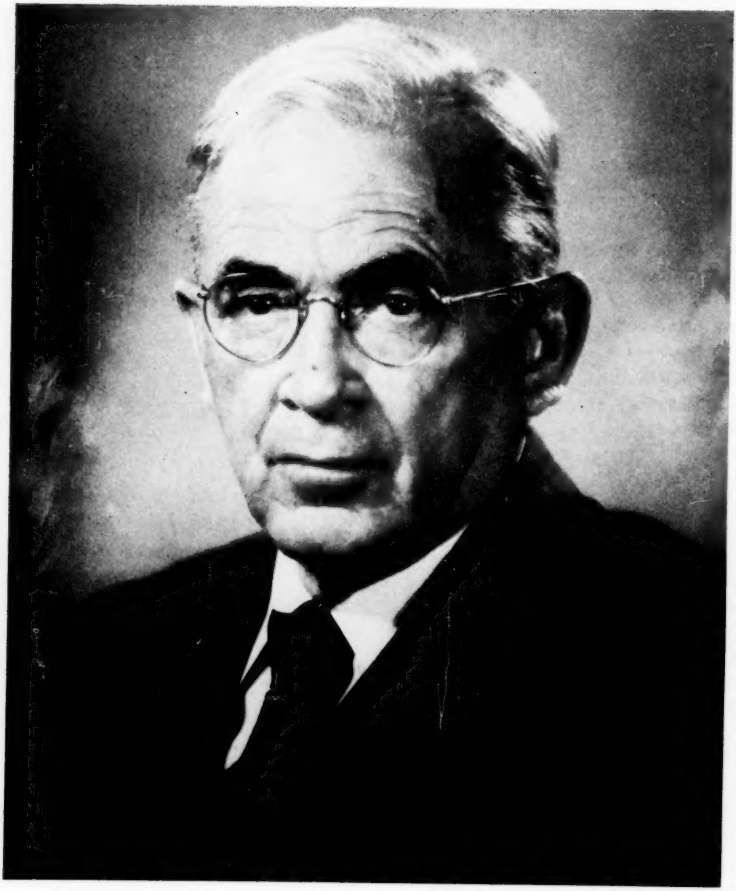

W. H. Chandler

PREFATORY CHAPTER
PLANT PHYSIOLOGY AND HORTICULTURE

By W. H. Chandler

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I completed my graduate training almost 50 years ago and have since been so largely concerned with the horticulture of trees that I do not feel competent to discuss plant physiology, as such, as it has developed during this past half-century. I hope, however, that reflections on selected field experiments that have served to translate laboratory research into practical horticulture may be of interest.

Horticulturists, like agronomists and plant pathologists, are now making extensive use of the discoveries reported from physiological laboratories. Horticulturists today are plant physiologists or geneticists who work mainly with horticultural species and maintain acquaintance with what is known about them from scientific study and from practice. They may or may not have obtained part of their college training in departments of horticulture. I think horticultural courses, especially seminars, would help them. But some men have become excellent horticulturists, who had their graduate training where there were no such courses, especially if they began work with good horticulturists. For example, some of the workers in tung research were trained as plant physiologists, but under the leadership of a good horticulturist they, too, soon became excellent horticulturists (50). Some may use this training mainly to study problems that are urgent to growers, but most find time to study for the purpose of increasing knowledge of the special nature and responses of horticultural species—knowledge that may be helpful toward the solution of problems which may arise in the future. Naturally, such studies add to the general knowledge of plant nature and responses.

Such close relationships between horticulturists and plant physiologists did not prevail 50 years ago, partly because physiologists were not making such useful discoveries and partly because horticulturists were less interested in such discoveries. Late in the 19th century when the American agricultural colleges were being established, teachers with special training to study crop species were not available. Much had been learned in Europe by observant fruit growers and nurserymen about responses of orchard species and the special responses of their varieties to environmental conditions and orchard and nursery practices. This knowledge had been increased by the observations of such men working in the climate of America. Books based on such observations had been published, among which was the one by J. J. Thomas which went through many editions (64). The early land-grant college horticulturists supplemented this knowledge by observations much like the ecological ones made by systematic botanists. They had little time or funds for

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well organized research and were too busy teaching students and growers what they thought were helpful principles. The practices they recommended were apt to be based on such physiological knowledge as they had and were sometimes inadvisable. They did not realize, I think, how much research would be required to explain the results of trial and error experience. New discoveries must be tried and adjusted cautiously before their practicability can be known. One of these early college horticulturists, however, found time for a contribution concerning the special nature of deciduous fruit trees. He made a careful microscopic study to determine the time in summer when flower initials can first be detected in buds of deciduous orchard species (31).

These early horticulturists were succeeded or joined by graduates of the colleges of agriculture, usually with little or no postgraduate training and not much interest in physiology. They planned their experiments largely to test orchard practices, especially the use of fertilizers and other soil treatments. They learned little about trees except that when comparing yield records of plants with different treatments the experimental error is very great. One worker (8, 9), however, had the good fortune to work with a soil so low in available phosphorus that grass and clover grew very weakly unless phosphorus was applied; yet apple trees, if adequately supplied with nitrogen, obtained enough phorphorus from the untreated soil to make healthy growth and give good yields. This evidence that deciduous orchard trees have a greater ability than grasses and some other field crops to obtain phosphorus from such soil has been supported by much additional experimentation. In the other experiments no such special characteristic of an orchard species was disclosed. In 1925 I reviewed the reports on all of these experiments—wisely, I then thought. But a member of an undergraduate class said that I had used 65 printed pages to say that if an element is deficient in the soil, fruit trees will respond to an application of that element if it gets into the roots. After some resentment and then reflection I realized that the student was right. The experiments had disclosed some differences among soils in their capacity to supply some of the essential elements. However, there was little evidence concerning: (*a*) special responses of orchard species to deficiencies or excesses of any nutrient except nitrogen, or (*b*) special ability, or inability, to obtain nutrients from difficultly soluble combinations in the soil.

Younger horticulturists with better training and with these experiments before them, are arranging their plots for better statistical analysis and, much more important, I think, are not relying so largely on yield records. They are instead, looking for special responses of trees of different orchard species to deficiencies of each nutrient (42). Since about 1925 considerable attention has been given to the symptoms displayed by different orchard species to each nutrient deficiency (68, 69). These are helpful, though not entirely dependable, since a deficiency may be great enough to cause reduction in growth and yield but not serious enough to cause the characteristic symptoms. Analysis of plant parts for the element being studied is proving helpful. Leaves of a standardized age-range and location on the tree are most apt to be

used, because adequate leaf samples can be taken without much physiological effect on the tree (18, 27, 53). Leaf analysis may not disclose deficiencies too slight to cause mild symptoms, but in standardized samples it may be more dependable than symptoms and seems to make it possible to locate areas of an orchard district where trees are certainly receiving enough of an element from the soil, where they are certainly deficient, and where there is doubt enough to make careful trials advisable. The leaf composition that indicates a barely adequate supply of an element may be influenced by above-ground features of the environment, such as temperature, light, or humidity and, therefore, may be higher in one district than in another (54). One of the most useful results of leaf analysis is the determination of whether or not an element applied to the soil gets into the trees. Fruit analysis may be as good for this purpose, but the evidence is meager (5, 40, 41). Fruit analysis may be the best way to determine whether or not an element sprayed onto the leaves gets into the tree effectively (40). Fertilizer sprays are being tried because some elements applied to some soils may be held above most of the roots, and because available combinations of nitrogen from soil applications may be most abundant in late summer when high nitrogen content may impair the quality of the fruit (40).

Examples of the amount of field study that may be required to get results of laboratory studies safely into practice are supplied by elements required in such small quantities that great care in the laboratory was required to prove them essential. By such care plant physiologists have learned that zinc, copper, manganese, boron, and molybdenum are essential for plants but in such minute quantities, that none of them would be expected to be deficient in a soil (39, 52, 58, 59, 60). Yet each has been found very injuriously deficient in orchards in some part of the world. For some of them many experiments were required to learn which element was deficient and even more to find a harmless and economical way to get the element into the trees. Thus, before 1935, millions of dollars worth of orchard trees were being killed or rendered worthless by a disease known as rosette on apple trees in Washington (47), as little-leaf on grapes and stone fruits in California (14), as yellows on walnuts, as mottle-leaf on citrus (17), and as rosette on pecan trees in southern states. On the basis of laboratory reports (32) and field studies in Florida (4, 10), all of the minor elements were applied to the soil of affected trees in experiments conducted in California. As much as 90 pounds per acre of zinc sulphate was applied which was more than the trees would use in 30 years. There was no response to any of these. Micro-nutrient deficiency appeared not to be the cause of peach tree little-leaf. By June, however, trees on open, sandy soil each of which had received in January 20 pounds or more of ferrous sulphate obtained from one supplier were showing striking improvement. Ferrous sulphate from other sources and chemically pure ferrous sulphate were without effect, as also were other substances that lowered the soil pH such as alum, aluminum sulphate, or sulphur. The effective ferrous sulphate was found to have been made from

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scrap iron with sulphuric acid and to contain a considerable amount of zinc. Trees were then treated by applying to the soil in winter 600 pounds of zinc sulphate per acre. By June these trees were showing striking improvement. By 1931 little-leaf of stone fruit trees was known to be caused by zinc deficiency (19, 20). But very much more study was required to identify, for the different orchard species, zinc deficiency symptoms on trees in different situations, and to get zinc into the trees safely and economically. Trials were gradually established in more than 50 orchards in 10 counties as symptoms were reported. No other district in California was found in which the application of 600 pounds of zinc sulphate per acre would cure the trees. In some soils, 2000 pounds per acre would cure some trees (particularly walnut) but not all of them; yet an ounce or two of zinc sulphate per tree plugged into holes about an inch and a half deep and four inches apart around the trunk in winter while the leaves were off would do so. This method, although effective, does not seem advisable, except for identifying symptoms, because of injury to the sapwood. Concentrating the zinc sulphate in a trench around the tree trunk so that the zinc would not have to pass through so much fixing soil to the roots reduced, a little, the amount required but not enough to make soil application economical. Even the acid, sandy soils of Florida citrus orchards have enough fixing ability to make soil applications expensive. In California a single soil treatment did not keep the trees healthy more than three to five years, if that long.

Spraying citrus trees with six to ten pounds of zinc sulphate and half as much lime or soda ash per hundred gallons of water annually or sometimes less often, supplied enough zinc. Spraying the foliage of peach or plum trees with such a mixture was not very effective. But spraying in winter with 10 to 40 pounds of zinc sulphate alone, or other soluble zinc compounds, per hundred gallons of water would cause: (*a*) healthy leaf growth from the first opening of the buds, and (*b*) usually the setting of a good crop of normal fruit from flowers which would fall from untreated trees or produce abnormal fruit. Improvement from zinc plugged into holes in the trunk did not show this early though it caused improvement a little sooner than soil treatment. This delay may have been caused, at least in part, by the deposition of zinc along the vessel walls before any of it reached the buds. Some recent studies with radioactive zinc indicated such deposits in vessels of grape vines (35). On young trees that make long late growth, and on older trees so deficient that many of the branch ends are dead, as much as 30 to 40 pounds of zinc sulphate per hundred gallons of water in a winter spray may be required, and even this may not get enough zinc into the tree to supply it to the end of such late summer growth. On such trees, another spray using zinc oxide—about six pounds per hundred gallons of water—in June or early July may be advisable, if little or no fruit is on the tree to be injured by the the spray. After trees have been kept healthy throughout one summer, a winter spray of 20 pounds or less of zinc sulphate per hundred gallons of water annually will tend to keep them healthy. Trees with the most dead

twigs caused by the deficiency required the most concentrated spray; on the other hand, the amount of spray required was unrelated to the fixing capacity of the soil (19, 20).

In the state of Washington, cherry and apple trees are now kept free of zinc deficiency symptoms by spraying after the crop is harvested but before leaf fall and again in the spring with three or four pounds of zinc sulphate alone per hundred gallons of water. Cherry trees, like walnut trees, do not respond well even to as much as 40 pounds of zinc sulphate per hundred gallons of water applied when the leaves are off (11).

Driving nearly headless, zinc-coated nails, an inch or more long, around and along the trunk or branches was effective in supplying zinc to trees of all deciduous orchard species, but 25 to 30 nails for each inch of trunk or branch circumference were necessary. Nails closer than a half inch apart in all directions may kill the bark between them. Walnut and cherry trees have been saved by this method, but labor costs are too great for general use. Because the nails can be driven safely at any time of the year, it is an excellent method of determining whether or not doubtful symptoms in an orchard are due to zinc deficiency (19, 20).

Chelated zinc compounds applied to the soil do not seem as effective in getting zinc into trees as chelated iron compounds are in supplying iron. In Florida zinc chelates penetrated the soil faster than zinc sulphate but the zinc did not get to the leaves any faster (61). In Washington, annual soil applications of about a pound of zinc chelate kept peach and sweet cherry orchards healthy, but did not seem to increase the zinc in the apple leaves (11). This type of application was too costly.

The studies in California gradually came to include many orchards because of the different symptoms displayed. Most of them proved to be caused by zinc deficiency. One of the last orchards studied showed symptoms of the smallest deficiency found. These trees grew vigorously and bore heavy crops of fruit of good market size, but much of this fruit turned brown internally before it could be canned. Careful study disclosed long water sprouts on which the latest growth at the tops showed small, closely-packed, distorted leaves with yellow splashes between the veins. All other leaves on the trees were without symptoms. An annual winter spray with 20 pounds or less of zinc sulphate per hundred gallons of water prevented this early browning of the fruit flesh.

When the deficiency is greater, symptoms on the water sprouts begin to show before they are so large. The first spring leaves on nearly all shoots may show yellow splashes between the veins, and some may be very small and stiff in closely packed rosettes on abnormally short shoots. Leaves formed a little later in spring may be free from mottling; those formed still later, on strong shoots, may be mottled and distorted. Thus, in midsummer a fairly strong shoot may have mottled leaves at its base, mottled and distorted leaves at its apex, and normal leaves between. If the deficiency is still greater, many shoots may die by autumn. In the following spring most

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of the flowers may fail to set fruit especially on apical parts of the branches. If fruits mature on these parts, they are small and abnormal in form. Branchlets on the lower parts of these main branches may bear fruit that is normal in size and appearance. Although mottling and rosettes are the most widely observed symptoms of zinc deficiency, weak trees bearing small, pale leaves with no distinct mottling or abnormality in form (leaves like those on trees made weak by nematodes or other root injury) sometimes show a striking response to zinc treatment. The only way to detect zinc deficiency in such orchards is to treat trees or branches with zinc and look for improved growth with better leaf size and color (19, 20).

Even in orchards where the soil normally supplies enough zinc, trees in an area that has been a feed lot in preceding years may show very severe zinc and copper deficiency injury. These feed lot soils in California are very high in potassium carbonate and in available phosphorus. Yet when either or both of these substances were applied to soils until its contents were as high as those of feed lot soils, the trees remained healthy and no zinc deficiency appeared during 12 years of observation (21). Results of heavy applications of phosphorus in causing zinc deficiency are conflicting (15). Possibly this may be explained by differences in amounts of such zinc-phosphorus compounds in the root zone; if absorbing parts of very many healthy roots are in contact with particles of such compounds, enough zinc may be dissolved at root surfaces for the small requirement of plants (21). Excess copper seems much stronger than excess phosphorus in its reduction of zinc intake (43).

In some Pacific slope soils on which trees showed zinc deficiency, crops such as alfalfa obtained enough zinc for excellent growth. In California, even maize leaves did not show the chlorotic streaks described for zinc deficiency in Florida (10). Alfalfa among the trees saved many zinc-deficient orchards in Washington before the cause of injury was known. This practice was not as effective in California. Trees in some walnut orchards were dying back from zinc deficiency with exceptionally vigorous growth of alfalfa among them. In some zinc-deficient orchards, however, benefit from alfalfa among the trees was obvious; in some, even weeds among the trees was clearly beneficial (19, 20). This effectiveness, partial effectiveness, and ineffectiveness of alfalfa, weeds, and other thick-growing plants in causing the soil to supply enough zinc to trees, may possibly be explained by the fact that in these semi-arid districts most of the zinc tends to be near the surface (36), and that this, when unshaded, may be at times hot enough to kill roots. If roots near the surface are killed or greatly weakened by nematodes or some other enemy, then shade from alfalfa or weeds will be less beneficial.

While the California studies were in progress, Florida workers were finding zinc deficiency injury in citrus and other orchards, following their earlier discoveries in field crops (4, 10); and in the Gulf states, zinc deficiency was found to be the cause of pecan rosette (2). After these reports, zinc deficiency was observed in plants in many parts of the world, but the symptoms were not usually sufficiently like those described for deciduous and citrus or-

chards that treatment could be predicted without careful field study (67). A considerable amount of laboratory study has concerned the possible role of zinc in plant processes. Some evidence indicates roles in enzyme activity such as production of tryptophane, a precursor of auxin (34, 48, 51, 57, 65). But nothing has been learned or seems likely to be learned that, if known earlier, would have made these extensive field studies unnecessary; furthermore, many millions of dollars worth of good orchards are now grown on land that would not grow good orchards before these field studies were made. In fact, after more than 50 years of contact with plant physiology, I do not remember a report of a laboratory study in that field that could be applied to the orchard without careful orchard study.

Among the most striking examples of the use in field studies of leads given by the laboratory, are trials of regulators, the use of which began as a result of the discovery of auxin (72). The activity of auxin was discovered as a result of tropism studies. About 40 years ago one great plant physiologist doubted the value of so much tropism study. Pfeffer (49) devoted 101 large pages to a review of tropism literature and 134 pages to related phenomena. Darwin (26) had devoted 573 pages to reports of such studies. Soon after indoleacetic acid became known as an auxin, a number of synthetic ring compounds with acetic, propionic and butyric acids were found to have similar effects on plants (37, 63, 74, 75). Such compounds have been called regulators, growth regulators, or growth substances, even hormones or auxins. Horticulturists in different parts of the world have made much use of these discoveries. One (66) has studied the movement of natural auxin in trees by which he seems able to control the spread of branches. Many have studied the different regulators as aids in vegetative propagation of plants in different species. One group of horticulturists (28, 29), finding that regulators applied to the bases of cuttings tended to delay abscission of petioles from which the leaves had been cut, tried them as sprays to reduce preharvest drop of fruits. Many other studies have followed so that the best and safest concentration and time of application of the best regulators is being learned for each important orchard variety in many different environments. Regulators used late enough to reduce preharvest drop tend to hasten respiration and maturity of fruit after harvest, especially of summer- and autumn-ripening varieties of apples (1, 33, 38) and of pears (3). Studies must, therefore, include tests for concentrations that are as effective as they can be without causing too rapid softening. On the other hand, successive regulator sprays on sour cherries caused the fruits to hang on until autumn and still be edible (73). A regulator dip (62) has been introduced into lemon packing sheds to delay dying of the calyx and to reduce injury by a fungus.

On figs of varieties that require pollination for setting fruit, some regulators caused setting without pollination. Some but not all, caused endocarps to develop and give something near the crunchy texture that the seeds give to pollinated fruit and that is wanted in markets for dried figs (23). Although they did not prove as satisfactory as pollination, sprays are known

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that probably could save the industry if some situation should arise that would make caprification (pollination) unsatisfactory.

For thinning fruit, rather extensive trials of regulator sprays two to four weeks after the bloom have tended to give promising results but disclosed many complications (38). For example, α-naphthaleneacetic acid applied less than 14 days after petal fall is apt to cause too much distortion of leaves. Applied later to trees of early-ripening varieties it may hasten ripening of fruit and cause it to be worthless. On the other hand, naphthaleneacetamide applied at blossoming time does not cause leaf distortion and, applied 10 to 14 days after flower opening, may cause seed abortion and hanging onto the tree of many small, worthless fruits. Much other detailed knowledge has been recorded of responses of varieties to different regulators and to time and concentration of application.

Regulators have been used to increase the size of fruit and to hasten ripening. Berry size and stem thickness of grapes (70) were increased by a spray of 4-chlorophenoxyacetic acid and drupelet size of blackberries (45) by a spray of this and other regulators at blossom time. In extensive trials with 2,4,5-trichlorophenoxyacetic acid ripening of peaches was hastened but in most cases size, color, and flavor of fruit were reduced (44, 71). On apricots, fruit drop was reduced, ripening hastened, and size of fruit increased by sprays with salts of 2,4,5-trichlorophenoxyacetic acid at about the time of pit hardening (24, 25). Fruit size increase was all due to increase in the size of cells. The volume of cell nuclei was greatly increased by the increase in number of chromosome sets (16). A spray with 2,4,5-trichlorophenoxyacetic acid, 16 hr. before a frost, caused the very tender tissue within the ovules of young fruits to have increased resistance to low temperature; and one given two days after the frost caused fruits with the tissue killed to develop to maturity instead of falling (24).

Injury from regulators may show in confusing ways. For example, in England, a spray with α-2,4,5-trichlorophenoxy propionic acid on Worcester Pearmain apple trees five or six weeks before normal ripening time, hastened ripening of the fruit 12 to 16 days. No injury to the trees was observed by leaf fall, but in the following spring bud opening was sparse and growth from those that did open was weak (1).

These have been only a few examples of much work by horticulturists with different regulators. By 1958 more than 250 papers had been published in the Proceedings of the American Society for Horticultural Science on the use of regulators, besides a considerable number of papers by horticulturists in botanical journals. Many papers reporting such research have been published in journals of other countries. The exacting choice of regulators and of concentrations and timing for different varieties and situations, and the confusing effects even among the few examples mentioned here seem to me to indicate that all of this study was necessary: to have recommended these substances widely on the basis of laboratory studies alone or of only a few

field trials could have caused serious losses and destroyed nearly all of the confidence growers may have in science.

The study of gibberellin is another example of the rapidity with which a new discovery now becomes tested on horticultural plants. Many horticulturists, especially workers with vegetables and flowers, are studying carefully the responses of plants to the gibberellins. Probably long before we know all of the roles this substance may have in plant processes, we shall known much about uses that can or can not be made of it in the culture of all horticultural species. And the laboratory physiologist may find help from these studies both in selecting the best varieties to work with and in new leads for his studies.

With horticulturists, as well as agronomists and plant pathologists, so alert for new help from the scientific literature, the plant physiologist and plant biochemist can work in their laboratories with assurance that any promise their discoveries may seem to have for plant culture will be tested carefully in so many environments that their usefulness and limitations will be known rather soon.

I do not mean to imply that a horticulturist works without a laboratory. For the trials mentioned here and for most of his field work, supplementary work in the laboratory may be required. He finds many of his best problems in the field, however, that is, special difficulties in special situations in commercial orchards. I do not mean that a person whose research and teaching concerns mainly some group of plants such as fruit trees or vegetable or field crops will obtain all or nearly all of his leads for research from such basic fields as plant physiology. Some will come from the experience of growers, more from the experience of the researcher. Such experience may give leads to plant physiologists for study, perhaps under better control, with any clone in the plant kingdom that seems most promising. For example, a very troublesome weed, the cocklebur (*Xanthium*) has been found to be one of the best species for the study of flower induction, but the lead for such studies came from a study of tobacco (30). Horticulturists have not merely supplied leads for more basic study by others; they have also made basic contributions published in horticultural periodicals and in journals of botany and plant physiology. However they need help from basic fields, especially if they work with such unwieldy material as fruit trees.

With the rapid growth of plant physiology and its increasing use of biochemistry and difficult techniques, the student preparing for horticultural research may not get the training that would help him most in seeking leads from the physiological literature. He will be wise, I think, to take all of the work he can get in plant physiology, including plant biochemistry, even if he must reduce the time he devotes to horticulture or prolong his graduate training. After he starts to work it will be easier for him to improve his training in horticulture than in such a broad field as plant physiology in which he will not be working daily. I would be doing many horticulturists

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an injustice if I assumed that work for a thesis with one of them would give a student no better training than the same work with me. But in my long teaching experience I have rarely been able to suggest to a student a problem in fruit tree physiology from which I thought he could gain as much in the time available, as he could gain from a problem using some other species. I have usually advised him to major in plant physiology and minor in horticulture taking our advanced courses and especially our seminars. Workers with vegetables, flowers, or harvested fruits (46, 55) could suggest more challenging problems that could be studied effectively in the time available. In fact, some of the most effective graduate training I have ever observed has been in a laboratory for the study of fruits, especially the avocado (6, 7, 12, 13, 22, 56). Plant science has made such great advances during the past 50 years, and work in progress promises so much more, that if a student is to become effective for work on the problems left, his thesis research should be chosen and studied in the full light of those advances.

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FOLIAR ABSORPTION OF MINERAL NUTRIENTS[1,2,3]

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The absorption of nutrients by various plant parts has been the subject of much research. In some instances the plant in its entirety, and in many other instances abscissed organs have been studied. All plant parts absorb from their environments water, solutes and gases. Losses of the same may occur from each part. The spectrum of materials known to be absorbed by plant foliage has become exceedingly broad. The phenomenon of foliar absorption, or the associated agricultural practice, has been described as foliar feeding, nutrient absorption by above-ground plant parts, extraradical feeding, nonroot feeding, and "Blättdungung."

Published reports on foliar absorption of mineral nutrients appeared as early as 1844 [Gris (38)] and were followed by those of Mayer (76) and Böhm (9). Research has been greatly facilitated since 1951 by the use of radioisotopes which permit accurate measurements of uptake and transport, and allow a distinction between nutrients absorbed by the foliage and those simultaneously taken up by the roots [Wittwer & Lundahl (145); Silberstein & Wittwer (108)]. Boynton's review (10) on foliar absorption of specific micro- and macronutrients has already appeared in this series. In the meantime, several summaries and reviews on foliar absorption of mineral nutrients have appeared from our group (127, 128, 130, 144, 147) and from abroad (63, 111, 115, 118, 120, 126). Other relevant reviews include those on absorption and transport of herbicides and growth regulators [Crafts (20); van Overbeek (135); Woodford *et al.* (148)] and on transport and redistribution of various substances in plants [Arisz (1); Williams (143)]. This review will include subject matter heretofore overlooked or unavailable, and present a summary of the most recent developments as they contribute to an understanding of the mechanisms of foliar absorption and transport, and related agricultural practices.

Mechanisms and Factors Affecting Absorption

Boynton (10) emphasized the importance of the contact angle of the applied solution droplets as well as surface wetting in foliar absorption. It is now apparent from many reports that surfactants seldom play a dominant role in mineral uptake (4, 60, 98, 114, 117).

[1] The survey of literature pertaining to this review was concluded September, 1958.

[2] This review was carried out as part of Contract AT(11-1)-158 with the Division of Biology and Medicine, U. S. Atomic Energy Commission.

[3] Journal Article No. 2335 from the Michigan Agricultural Experiment Station.

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Much evidence, some conflicting, for stomatal versus cuticular absorption was also presented (10). Passage through imperfections in the cuticular layer or through the cuticle itself rather than stomatal entry provides the only means of resolving the data now recorded (29, 40, 113, 117, 140). Indeed, according to Scott's observations (102), an internal cuticle ("suberin") covers the inner walls of leaf epidermal cells as well as the walls of the mesophyll cells of stomatal cavities. Thus, stomatal entry would in no way preclude the necessity of nutrients eventually passing through a cuticle.

Mention should be made of the detailed studies of Skoss (109) and those of Orgell (89, 90), on the nature of isolated leaf cuticles from a limited number of plant species. Skoss (109) has suggested, " . . . that stomates act as the major portal of entry, regardless of the nature of the sprayed substance." However, in the *in vivo* studies, only oils and herbicides were employed using physiological responses as criteria of penetration, and the species tested had leaves with a thick "waxy" cuticle (109). Excised cuticles from leaves of *Hedera helix* were impermeable to 2,4-dichlorophenoxyacetic acid, dinitro-*o*-secondary butylphenol, and to silver and chloride ions. When the wax was removed, the residual cutin was highly permeable to water. The problems involved in extending results obtained with readily isolated thick "waxy" cuticles to species from which only thin or fragmentary cuticle can be obtained have been recognized by Orgell (89). He has suggested that cracks and imperfections in the cuticle, or an imbricated cuticle of small platelets cemented together by pectic materials, may result in ready penetration of foliar-applied polar substances (89). This would constitute "intercuticular passage." However, the sorptive characteristics of the cuticle described by Orgell (90) as a semilipoidal cation exchange membrane are reminiscent of those of a selectively permeable amphoteric membrane (31) indicating the possibility of "intracuticular penetration." The relative sorptive capacity of an isolated cuticle for acidic substances decreases markedly from pH 1 to 7, and increases for basic substances from pH 3 to 9 (90). This, in fact, shows a striking similarity to the effect of pH of the applied solution on the relative rates of absorption of phosphate and potassium or rubidium (117). For phosphate a bimodal curve of uptake is obtained, probably as a consequence of differential sorption of two ionic species (117). Sorption by the cuticle would, however, constitute only the initial step in the overall process of nutrient uptake. In subsequent steps subcuticular epidermal plasmodesmata (67, 101) or "Ecktodesmata" (100) may conceivably perform an important function.

The evidence, at present, is insufficient to define completely the mechanisms involved in foliar absorption of individual nutrients. Yet, it is apparent that the presence of a semilipoidal cuticle in no way precludes the absorption of ionic substances since this layer occurs on all root hairs and on the outer epidermal walls of young roots (103) in that region from which maximum translocation occurs (62). As with root absorption (26), it might

be expected that the mechanisms of foliar absorption and factors controlling rates of uptake vary with the nutrient applied and the species under observation. Accordingly, the authors have elected to discuss foliar absorption from the standpoint of various nutrient groups involved.

Urea nitrogen.—Urea nitrogen is absorbed, transported, and metabolized as rapidly as any nutrient applied to plant foliage. While it presently constitutes the most widely used material in nutrient sprays, it is somewhat surprising that so little is known concerning the mechanisms of absorption and utilization. Extensive absorption and distribution throughout the plant within 24 hr. have been shown by the use of N^{15}-labeled urea applied to sugar cane (16) and tobacco (138). Uptake was most rapid at night and during the early morning hours (30, 138). A marked dependence of absorption on high relative humidity was evident (138). Data on wetting agents (98), temperature, and light (138) as they influence absorption of urea were not conclusive. The absorption of urea from buffered solutions was a function of both pH and the buffer system used (138). With various buffers absorption rate maxima occurred at pH 5 and 8 with minima at pH 6 and 9. It is now established that sucrose and some other sugars (25, 49, 51, 98, 106), as well as magnesium salts (28, 83) employed as "safening agents" to reduce foliar injury from urea, depress the rate of uptake (28, 51, 83, 98, 106), rather than reduce urease activity which has been suggested as a limiting factor in uptake (50).

Anions (phosphorus, sulfur, chlorine, iodine).—A pH of 2 to 3, as compared to a higher pH, of the applied phosphate solution, facilitates more rapid uptake by leaves (28, 108, 114, 117). Teubner *et al.* (117) have discussed this fact in terms of possible exchange reactions involving the ionic species of phosphate. In view of the effects of pH on the sorptive capacity of the cuticle (90), the penetration of undissociated molecules of H_3PO_4 at low pH levels may not play an important role. The mono-ammonium salt at low pH values has given maximum absorption (24, 82, 99, 117) in most instances, although Koontz & Biddulph (60) procured most rapid phosphate uptake with NaH_2PO_4. The addition of surfactants and wetting agents has variously increased (28, 117), decreased (114, 117), and had no effect (4, 60, 117) on uptake, depending on the plant species and the chemical nature and concentration of surfactant. Inconclusive results of a similar nature have been reported for the effects of temperature, humidity, light, and sucrose (4, 117, 120). However, when plants are grown in the dark to deplete their carbohydrate content, an energy source, e.g., glucose or fructose, may be essential for uptake (151, 157), particularly in those cases where subsequent transport from the site of application is limiting (4, 46, 117).

Sulfur, chlorine, and iodine are readily absorbed by plant foliage but factors controlling uptake have received little attention. The absorption of of sulfur by the foliage of cotton from SO_2 fumes in the atmosphere has recently been investigated by Olsen (88). Other pertinent papers have been

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reviewed by Boynton (10) and Tamm (115). The distribution patterns of absorbed S^{35} as $SO_4^=$ and Cl^{36} as Cl^- by bean leaves are similar to those for P^{32} (7, 13, 149). Unlike the situation with respect to phosphate, pH has little effect on uptake of sulfate (7). Reports by van Lookeren Campagne (134) and Arisz (2) suggest a light-dependence for chloride uptake by the leaves of *Vallisneria*. The action spectrum for chloride absorption was the same as that of the absorption spectrum of the chlorophyll system; however, the phenomenon also occurred in the absence of carbon dioxide (134). Thus, absorption of chloride in this species is an active process requiring light or another energy source, and is inhibited by azide, cyanide, arsenate, and uranylnitrate (2). Finally I^{131} both as I_2 vapor and I^- (sodium salt in solution) were absorbed by bean and geranium leaves after which rapid distribution throughout the plants occurred (104).

Monovalent cations (potassium, sodium, rubidium).—With the exception of urea and possibly some nitrogen salts, potassium, sodium and rubidium are the most readily absorbed of the mineral nutrients by the leaves of plants (13). Most rapid uptake of K^{42} and Rb^{86} occurs at a pH of 8 when applied as the phosphate or citrate salts (117).

Divalent cations (calcium, magnesium, strontium, barium).—Isotopic labeling has made possible a study of factors affecting absorption but little has been accomplished with the alkaline earth elements. While calcium as the chloride or nitrate salt is sprayed on some crops to control physiological disorders (35, 36), comparative rates of uptake are not known nor have the effects of pH or wetting agents been studied. The time of day was important with respect to absorption of magnesium by apple leaves (87). It is difficult to decide whether the evening applications were more effective because of the higher humidity during the hours that followed, or whether the hydrogen ion exchange hypothesis of Oland & Opland (87), involving a diurnal fluctuation in organic acid content, was operative. Data for magnesium, calcium, strontium, and barium suggest an initially rapid absorption by plant foliage, which decreases sharply after the first few hours (13, 28, 87). The rapid saturation of leaf tissue in the immediate area of treatment, accompanied by failure of the materials to be removed from beneath the treating sites may account for this effect (5, 13, 74). Chelation of magnesium (139) and strontium (74) did not facilitate absorption or transport. Salts of the divalent cations may be applied to plant foliage at considerably higher concentrations than are tolerated for other salts (28, 35, 36, 53, 74).

Trace elements (iron, manganese, zinc, copper, molybdenum, cobalt).—Absorption of the trace elements is rapid following foliar application, with a sharp decrease in absorption rate after a few hours (13). Limited mobility of these elements (5, 13) and accumulation in the leaf may be contributing factors in retarding absorption. High humidity has been reported as favoring absorption of manganese by soybean leaves (78). Cobalt is absorbed in either light or dark but is translocated only in light or in the presence of sugars

(39). It is regrettable that, with the availability of radioisotopes as tracers, and the agricultural importance of trace elements in foliar treatments for the prevention or cure of nutritional disorders, more is not known of the mechanisms and factors influencing absorption by aerial plant parts.

General considerations and interpretations.—A disturbing feature of most experiments on foliar absorption is that uptake and transport have not been clearly differentiated. Attempts at experimental resolution of the two processes leave much to be desired. The latest "washing after treatment technique" (4, 28, 120) has inherent sources of error for nutrients which are as strongly adsorbed as phosphate and for those which are as easily leached as potassium (72, 112). Temperature optima for absorption of some minerals by a few species have been established, and Q_{10} values approximating 2 have been reported for phosphorus (110), for phosphorus, potassium, and rubidium (117), and for manganese (78). These workers, however, did not distinguish between absorption and subsequent translocation. According to Barrier & Loomis (4), P^{32} absorption by soybean leaves showed no temperature effect, but their conclusions were based on a single comparison of absorption at 15° and 30°C. Teubner *et al.* (117) indicated that 21°C. constituted a temperature optimum for utilization (absorption and transport) of foliar applied phosphorus with Q_{10} values of 2 or higher at temperature ranges of 10° to 15°C.

It has been reported that plants deficient in phosphorus absorb foliar-applied P^{32} more rapidly than those grown in phosphorus-rich media (48). It is true that high nutrient intensities and high levels of phosphate in root media depressed the percentage of phosphate in the roots and fruits derived from foliar applications (3, 146). Yet, the absolute amounts of phosphate absorbed were often greater because of greater retention by a larger leaf area (117, 146). High levels of phosphate in the vascular system may also retard transport from the foliage rather than influence absorption.

There is almost universal agreement that foliar absorption rates for most nutrients are greater for young leaves than for old. These nutrients include urea (18, 138), phosphorus (28, 60, 82, 120), magnesium (87), potassium (66), zinc (113, 140), and manganese (78). A possible exception is potassium absorption by grape leaves (68).

Comparative absorption rates, expressed as time required for 50 per cent absorption of various nutrients applied to leaves of plants, are summarized in Table I. The slope of absorption curves from which many of these data were taken show a direct proportionality to the amount remaining on the foliage.

The mechanisms of foliar absorption based on the criteria in Table II can be defined only in part and only for a few of the nutrients. The primary mechanism for urea absorption is probably diffusion since it is absorbed and moves throughout the plant very rapidly (Table I). Furthermore, for urea uptake an energy source is apparently not required (25, 49, 51, 98, 106), and

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TABLE I

ABSORPTION RATES FOR NUTRIENTS APPLIED TO PLANT FOLIAGE

Element	Plant Treated	Time Required for 50% Absorption	References
		(Hours or days)	
Nitrogen (as urea)	Apple	1 to 4 hr.	Fisher & Walker (28)
	Pineapple	1 to 4 hr.	Sanford *et al.* (98)
	Sugar cane	<24 hr.	Burr *et al.* (16)
	Tobacco	24 to 36 hr.	Volk & McAuliffe (138)
	Coffee, cacao	1 to 6 hr.	Cain (18)
	Banana	1 to 6 hr.	Cain (18) Freiberg & Payne (30)
	Cucumber, bean, tomato, corn	1 to 6 hr.	Hinsvark *et al.* (50)
	Celery, potato	12 to 24 hr.	Hinsvark *et al.* (50)
Phosphorus	Apple	7 to 11 days	Fisher & Walker (28)
	Bean	30 hr.	Koontz & Biddulph (60)
		6 days	Bukovac & Wittwer (13)
	Sugar cane	15 days	Burr *et al.* (17)
Potassium	Bean, squash	1 to 4 days	Mayberry (75)
	Grape	1 to 4 days	Lecat (68)
Calcium	Bean	4 days	Bukovac & Wittwer (13)
Magnesium	Apple	20% in 1 hr.	Fisher & Walker (28)
Sodium	Bean	6 hr.	Bukovac & Wittwer (13)
Sulfur	Bean	8 days	Bukovac & Wittwer (13)
Chlorine	Bean	1 to 2 days	Bukovac & Wittwer (13)
Iron	Bean	8% in 24 hr.	Bukovac & Wittwer (13)
Manganese	Bean	24 to 48 hr.	Bukovac & Wittwer (13)
	Soybean		Mederski & Hoff (78)
Zinc	Bean	24 hr.	Bukovac & Wittwer (13)
Molybdenum	Bean	4% in 24 hr.	Bukovac & Wittwer (13)

the temperature coefficient (Q_{10}) for absorption is close to one (65, 128). A secondary (active) mechanism which may limit the rate of absorption in some species may be enzymatic hydrolysis at the leaf surface by urease (50). Judging from very limited data for accumulation (2, 13, 60), irreversibility (2, 131), pH responses (114, 117), sensitivity to inhibitors (2), and possibly

TABLE II

MECHANISMS OF ION UPTAKE BY LEAVES*

Criteria	Mechanisms		
	Active Absorption	Ion Exchange	Diffusion
Accumulation	Yes	Possible	No
Reversibility (Leachability)	No	Yes	Yes
Rapid Equilibration (Absorption Rate)	No	Yes	Yes
Sensitivity to Inhibitors	Yes	No	No
pH Dependence	Possible	Yes	No
Specificity and Ion Competition	Yes	Yes (Lyotropic Series for Cations)	No
Oxygen Tension	Yes	No	No
Energy Source (Carbohydrate or Light)	Yes	Indirect	No
Temperature Coefficient (Q_{10})	>2	<2	<2

* Modified after Epstein (26) for mechanisms of ion uptake by roots.

temperature effects (4, 110, 117, 120) and energy requirements (151, 157), either an exchange or an active absorption process appears to predominate for phosphate, sulfate, and chloride. Rapid absorption (equilibration?) rates (13, 28, 87) and reversibility as indicated by leaching studies (72, 112), together with some evidence of pH dependence (117), suggest the participation of ion exchange in the foliar absorption of sodium, potassium, calcium, and magnesium.

Great gaps exist in our knowledge of foliar absorption of all nutrients

with respect to many criteria listed in Table II. This is particularly true of the cations and trace elements where the effects of pH and ion competition would be most critical. Furthermore, no information is available for any nutrient with respect to specificity and oxygen tension. Knowledge of the effects of metabolic inhibitors is limited to chloride absorption and transport (1, 2) and to the transport of phosphate (56). Data on the reversibility of uptake are for the most part indirect in that they have been obtained largely from leaching studies where the nutrients were root absorbed (72, 80, 131). The effects of temperature and an energy source still need to be resolved even for urea and phosphate, which have been studied most extensively, since the effects on absorption have not been entirely distinguished from those on transport (4, 117, 120, 138, 157).

Transport and Mobility

In an extensive mobility study by Bukovac & Wittwer (13) rubidium, sodium, potassium, phosphorus, chlorine, and sulfur were classified as mobile; zinc, copper, manganese, iron, and molybdenum as partially mobile; and magnesium, calcium, strontium, and barium as immobile elements. This grouping is in agreement with the several reports of Biddulph & his co-workers (5 to 8) for phosphorus, sulfur, calcium, and iron, and with Woolley *et al.* (149) for chlorine.

There seems little doubt that transport of nutrients from the foliage is initially in the phloem (8, 150). This may be considered an "active" process since transport of phosphorus (4, 46, 117, 151, 157), cobalt (39), or chloride (1) requires an energy source (light or sugar). Metabolic inhibitors such as dinitrophenol and sodium fluoride retarded the transport of phosphate (56), while azide inhibited the transport of chloride (2). Swanson & Whitney (114) and Teubner *et al.* (117) found that low temperatures greatly retarded the transport of foliar-absorbed phosphate. These factors all suggest that an active or metabolic translocation is involved in certain instances, although diffusion may be a primary factor in others (1).

Special problems in mobility have appeared and their resolution has been intriguing. Calcium is normally translocated solely in an acropetal or polar direction (5, 13, 14, 74, 86). The nonpolar and downward transport of calcium in bean plants has been induced by mild anesthetization with diethyl ether (14); similar transport of both calcium and iron in tomato, peach, and apple has been induced with 2,3,5-triiodobenzoic acid and to a much lesser extent with maleic hydrazide (57). These chemicals interfere with the polar transport of auxins, affect apical dominance, and suspend polarity.

Kessler & Moscicki (57) have also reported that 2,3,5-triiodobenzoic acid promoted chlorophyll formation presumably through the release of immobilized iron. Attention is directed to a similar report by Samish (96) in which iron chlorosis in grapes was alleviated to some degree by sprays of either

iron or naphthaleneacetic acid, but much more so when the two were applied in combination.

Transport forms of foliar-applied nutrients have attracted but little attention. However, it is reported that following foliar absorption of P^{32} considerable amounts of labeled organic phosphates occur in the leaves (4, 110, 152), and that sugar phosphate esters play a dominant role in transport (6, 150, 153).

Our analyses of the phosphate fractions in bean stem tissue 2 hr. after foliar application of labeled phosphate show that up to 90 per cent of the radioactivity in a stem segment occurs as inorganic phosphate (117). However, with a time interval of only 30 min. between treatment of leaves and harvest of stems, 20 per cent of the radioactivity is found in the inorganic and over 50 per cent in the hexose-phosphate fraction. It was suggested that initially large quantities of hexose phosphates are synthesized in the leaf following foliar absorption but phosphorus was transported primarily as inorganic phosphate (117). This is in agreement with the observation of Tolbert & Wiebe (125) that foliar-applied P^{32}-labeled phosphate in phloem exudates of squash plants occurs primarily as inorganic phosphate.

Metabolism of Foliar-Applied Nutrients

Urea.—The metabolism of foliar-applied urea has been studied extensively and the early work reviewed by Boynton (10). Hinsvark *et al.* (50) employed foliar-applied C^{14}-labeled urea and measured the evolution of $C^{14}O_2$ from treated bean, tomato, cucumber, corn, celery, and potato plants in a closed system. They concluded that hydrolysis to CO_2 and NH_3 by the enzyme urease was a rate-limiting reaction in the absorption and utilization of the nitrogen. In contrast, Webster *et al.* (141), using C^{14} urea and other C^{14}-labeled compounds, and following the fate of the C^{14} in each, concluded that urea was absorbed more rapidly than it was hydrolyzed by bean leaves. In this case, urea hydrolysis was a rate-limiting factor for glutamine and asparagine synthesis. Studies on detached citrus leaves by Kuykendall & Wallace (65) suggested, however, that urease activity is sufficiently great that the rate of urea hydrolysis should never be a limiting factor in the assimilation of foliar-applied urea. In this connection, Freiberg & Payne (30) report that no urease activity occurs in the banana leaf, but as much as 65 per cent of the applied urea is absorbed within 25 min. Hydrolysis of the C^{14}-labeled urea occurred in the growing points which were the only vegetative regions in which urease activity was detected. According to Cain (18) for coffee and banana and Malavolta *et al.* (73) for coffee, absorption is followed by a rapid disappearance of urea and an accumulation of amino acids with asparagine and glutamic acid predominating. Similar results have been reported by Japanese workers (155, 156). Workers at the Pineapple Research Institute (98) report a rapid uptake of urea at night with conversion to ammonia. Subsequently, during the day, glutamine accumulates. The rapid

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distribution and conversion of nitrogen from foliar-applied urea to useful metabolic components in the pineapple plant is evidenced by the "greening up" of the entire aerial parts when urea is applied to only a small part of the total leaf surface (98).

It is of interest that biuret was discovered as the toxic ingredient in certain urea formulations applied to the pineapple (97). The nature of this biuret toxicity, however, in terms of plant metabolism has not been established. It is apparent that many more plant species should be examined for urease activity, and urea hydrolysis studies correlated with accurate determinations of uptake using both C^{14}- and N^{15}-labeled urea. The possibility of direct metabolism of urea, bypassing enzymatic hydrolysis to ammonia, should also be investigated.

Phosphorus and sulfur.—A rapid (20 to 30 min.) incorporation of a part of the foliar-applied P^{32}-labeled phosphate into sugar phosphate esters with fructose-1,6-diphosphate predominating has been reported for bean plants (6, 153). Utilization of foliar-applied inorganic phosphate in the sugar, lipide and protein metabolism of the plant has also been demonstrated (4, 110, 117, 150). There seems little doubt that foliar-absorbed phosphate and sulfur (133) are readily converted to complex organic fractions in the leaf.

Leaching of Nutrients from Plant Foliage

Studies of the reversibility of nutrient uptake by leaves have followed closely the research on foliar absorption. As with many recent leaf-absorption studies, radioisotopes have proved useful as tracers (72, 80, 131). Simple diffusion and ion exchange mechanisms (Table II) could easily account, in most instances, for the loss of nutrients through leaching of plant foliage by rainfall or heavy dews, provided these nutrients occur in appreciable quantities outside the plasma membrane, or the retentive ability of the cells is in some way impaired. Ions adsorbed on the cuticle (90) could exchange for H^+ and HCO_3^- in carbon dioxide-saturated rainfall. Any active mechanism of relieving plants of excess salts is probably confined chiefly to the Plumbaginaceae and Frankeniaceae (45) in which salt glands occur. However, guttation is a widespread phenomenon among plants and the presence of both organic and inorganic salts in guttation liquids is well established (112).

Stenlid (112) has confirmed that, of all nutrients, potassium is the most easily leached from plant foliage by rainwater. This was indicated in isotope studies by Long and his co-workers (72). After studying leaching of radioisotopes previously absorbed through the roots and translocated to the leaves, Tukey *et al.* (131) have listed Na^{22} and Mn^{54} as most readily leached from young foliage of herbaceous plants, followed by Ca^{45}, Mg^{28}, K^{42}, S^{35}, and Sr^{90} which were moderately leached, while Fe^{55-59}, Zn^{65}, P^{32}, and Cl^{36} were leached with great difficulty. Ease of nutrient removal from leaves does not parallel absorption rates, since nutrients absorbed with similar rapidity (i.e., Na^{22}, K^{42}, and Cl^{36}) were leached at different rates. Also, in contrast to

absorptive phenomena, leaching from old leaves and upper leaf surfaces is usually much greater than from young leaves and lower surfaces (112).

Mineral losses from aerial parts of apple trees, even during winter dormancy, suggest that nutrients are leached by rainfall from structures other than leaves. Losses, however, are much less than from the foliage (21).

It seems clear that too little attention has been paid to the physiological importance of the phenomenon of leaching. In many greenhouse nutritional experiments, the possibility of its occurring is often eliminated by the environmental controls imposed (132). Under natural growing conditions, however, it may prove to be an important factor regulating the organic as well as the mineral composition of aerial plant parts (21).

Crop Response

Pineapple.—Seventy-five to 80 per cent of the nitrogen applied to Hawaiian pineapple fields is in the form of urea sprays, and 40 to 50 per cent of the phosphorus and potassium fertilizer is applied to the foliage according to Sanford and co-workers (98). Some plantations use urea sprays exclusively as the source of nitrogen. Nutritional spraying of pineapple has developed because (*a*) foliar applications were found fully as effective as soil treatments; (*b*) the pineapple leaf can tolerate high concentrations of dissolved nutrients; and (*c*) trace elements are regularly applied and the major elements, particularly urea, simply take a "free ride" (98). There is little doubt but that foliar absorption is involved since "low gallonage" (less than 50 gallons per acre) spraying, where there is no runoff, is fully as effective as the "high gallonage" treatment (98).

Sugar cane.—The extent to which foliar feeding is currently practiced for sugar cane production is not clear from published papers, but it is reported (17) that high percentages of the required nutrients can be supplied by foliage sprays. Burr and his co-workers (15, 16) have reported that rapid uptake of N^{15}-labeled urea occurred through the leaves and that within 24 hr. it was transported throughout the plant, even to roots which were bathed in a culture solution high in nitrogen. Other studies with P^{32}, Rb^{86}, and C^{14}-labeled sucrose (16) have led the same group to conclude that these materials are readily absorbed by the leaves and distributed throughout the plant. Absorption curves were logarithmic with half-times ranging from one day for sucrose to about 15 days for P^{32} and Rb^{86}. Phosphate absorption was more rapid from the lower leaf surfaces, was aided by a surface active agent, and ammonium phosphates were absorbed more readily than the potassium salts. Calculations were provided showing that the entire phosphorus requirement of the sugarcane plant may be supplied through the leaves (16).

Tree fruits.—Foliar applications of urea, magnesium, and certain trace elements serve as effective and efficient means of supplying or supplementing the nutritional requirements for these elements of tree fruits, including citrus. Recent studies with urea have shown that apples respond, but some varieties

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more than others. On the other hand, pears and stone fruits have exhibited little benefit (93, 94). Failure of stone fruits and pears to utilize effectively foliar-applied urea has not been resolved. Either the mechanisms of absorption or hydrolysis, or both, may be limiting.

Chelated magnesium was less effective (139), and the nitrate and chloride salts were more rapidly absorbed by apple leaves than was the commonly used magnesium sulfate (28). A similar advantage of the chloride over the sulfate salt of magnesium has been reported by Hagler (41).

Phosphorus is required by tree fruits only to the extent of about one-tenth that of nitrogen (93). Since it is also readily and efficiently absorbed by the foliage (24, 28), it has potential use in nutritional spraying. Fruit tree responses to foliar-applied phosphorus have not been great (93) nor do fruit trees normally respond to soil applications (94). Since the need for phosphate is seldom critical and, unlike urea, it may react with other spray materials to give insoluble forms or release toxic compounds (93), its inclusion in a normal spray program may not be warranted.

Potassium sprays have not generally proved useful on woody plants because of the large amounts needed and the foliage injury encountered when soluble salts are applied. However, very concentrated solutions (32 per cent) of potassium nitrate may be applied to dormant apple and cherry trees without injury (127). Venkataramani (136, 137) obtained striking increases in leaf yields of "debilitated" tea plants following foliar applications of KCl, equivalent to 52 pounds of K_2O per acre per year, with no additional gain when nitrogen and phosphorus were added.

Zn^{65} absorption and penetration through citrus foliage was a function of the concentration applied per unit area, and final distribution was similar to that absorbed by the roots (140). Heymann-Herschberg (47) has reported that zinc sulfate and sulfur sprays (or dusts) may be combined to control both citrus rust mite and zinc deficiency in orange trees.

Aerial parts other than leaves also function in nutrient uptake. C^{14}-labeled urea, P^{32}, K^{42}, S^{35} and Fe^{55} have been absorbed through the bark of deciduous fruit trees (43, 69, 127, 129), and zinc through the bark of citrus (113). It appears that some active growth is necessary for most rapid uptake, and entry is facilitated by pruning wounds, injuries, and other imperfections (43, 124). Bark absorption of nutrients is not unlike that which occurs through the suberized roots of many tree crops (62). The extent to which concentrated nutrient sprays, applied to woody plants at dormant or "delayed dormant" stages, contribute to the total nutrition of the plant has not been determined. Here again isotopic labeling can be employed to great advantage.

Vegetable and agronomic crops.—While under some conditions sprays of trace elements have corrected nutritional disorders (70, 71), many equally beneficial effects of leaf feeding have occurred on vegetable crops with the immobile macro-elements. Sprays of calcium effectively control blackheart

in celery (35) and blossom-end rot in tomatoes (36). Magnesium sprays on celery grown on muck soils have alleviated a deficiency that was impractical to control by soil treatments (53).

It has been shown conclusively that nitrogen, phosphorus, and potassium, are readily absorbed by aerial plant parts, often several times more efficiently than from supplementary soil treatments (28, 108, 119, 121, 123, 146). Yet, there are few examples which show positive yield or growth responses above those which could be procured by the most effective soil fertilization practices (11, 44, 77, 79). Numerous experiments with soil applications of radiophosphorus to agronomic crops have been summarized by Dean (22), showing that the greatest need for fertilizer phosphorus is during the early stages of growth. At this time leaves are small and few in number, and the surface areas grossly inadequate for the absorption of nutrients from foliar sprays as far as total needs are concerned. The benefits from foliar fertilization of row crops have come chiefly when sprays were applied after extensive or maximal leaf areas had developed, and then repeat treatments were often necessary (44, 55, 77, 122).

Nevertheless, nutritional spraying of row crops and small grains with complete fertilizers has developed as an agricultural practice and has now achieved considerable prominence abroad. Numerous technical as well as practical publications (12, 19, 23, 27, 32, 33, 34, 61, 64, 84, 85, 92, 105, 119, 155) have reported beneficial results if treatments are made during the later stages of vegetative development or during early flowering and fruiting. In this connection Kaindl (54, 55) logically postulated that the greatest benefits from foliar feeding of phosphate would occur with the small grains just before and at heading, when leaf areas are large and plants have "maximum vitality." A similar concept is proposed by Thorne & Watson (122) for nitrogen applications on wheat. Still lacking, however, are accurate determinations of the nutrient contributions to edible parts and plant residues. Some preliminary field studies with P^{32}-labeled *o*-phosphoric acid show that from 10 to 14 per cent of the phosphorus in the edible portions of potatoes, tomatoes, corn, and beans at harvest may be derived from four foliar sprays applied at weekly intervals during flowering and fruiting (116).

Discussion

Foliar absorption and its reversible counterpart, "leaching," constitute two important processes in the control of salt metabolism and moisture relationships of plants grown under natural conditions. The absorptive capacities of above-ground parts are being used in the application of numerous plant nutrients and agricultural chemicals. Foliar absorption is also significant in that the atmosphere (115) may be a major source of certain elements. Gases, fumes, dusts, and water—both rainfall and irrigation—which contact leaf surfaces contribute to, or reduce, the salt content of plants. Several vegetable crops and pineapples grown near the sea upon which sprays of salt water fall

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periodically contained several times the normal amounts of sodium and chlorine (107, 142).

Similarly, citrus leaves absorb large quantities of salts from sprinkler irrigation water (42). Atomic fall-out components deposited on plants as dust particles or in rainfall are readily taken up through the foliage and other above ground parts (37, 58, 95, 154). Radiostrontium (Sr^{89} and Sr^{90}) and ruthenium (Ru^{103}) are absorbed, but with little movement from the absorbing organs (52, 59, 74, 81). Caesium (Ce^{137}), however, is mobile and moves throughout the plant (59, 81). The significance of foliar absorption of "fall-out" by food crops near areas of atomic blasts is apparent from reports of high levels of radioactivity in the tops but little if any in the roots of test plants (95). Absorption and distribution throughout the absorbing organ (52, 74) preclude the decontamination of many fruits and vegetables by washing or peeling.

An increase in availability of nutrients for plants grown in cold climates may extend the boundaries of successful crop production. Air temperature, rather than soil temperature, controls uptake and transport of nutrients applied to the foliage. A great differential in soil and air temperatures occurs in early spring as well as in the perma-frost agricultural areas of the far north. Under these conditions foliar applications of nutrients should promote earlier growth and increase productivity. This has, in fact, already been demonstrated by preliminary studies of phosphate utilization of crops in the Soviet Union (158) and by limited field tests in the spring with strawberries in Michigan (86).

Except for the pioneering studies of Pastac (91), optimum formulations and ratios of nitrogen, phosphorus, and potassium for foliar sprays have apparently attracted little attention. Yet the most effective ratios may differ greatly from those which are most useful as soil treatments. Rates of foliar absorption and transport vary with each nutrient, and applying nutrients to aerial plant parts avoids the fixative and exchange capacities of the soil.

A cuticular barrier in the penetration of solutes appears almost certain from the evidence that lightly cutinized young leaves absorb nutrients faster than the heavily cutinized old leaves, and lower leaf surfaces are often more efficient than the upper. The analogy fails to hold, however, in a consideration of nutrient removal where leaching by rainfall is much greater in old leaves and from upper leaf surfaces. The postulation of a predominating active absorption and transport mechanism (Table II) in young leaves which is largely irreversible both within the plant (60) and to the external environment provides an alternate explanation of the facts thus far assembled. Mechanisms of active uptake, ion exchange, and diffusion may be essentially the same for absorption by aerial plant parts as for roots. Cuticular barriers on leaves and stems are probably of no more consequence than those on root surfaces (62, 103).

However, other problems of foliar absorption—or at least their resolution

—are different than those encountered in root absorption. In the usual nutritional experiment, roots are continuously immersed in a solution, the components of which may be regulated at will and the root environment easily standardized. When nutrients are applied to leaves, far greater concentrations are employed than can be tolerated by roots. Evaporation of the aqueous phase of these solutions usually occurs within minutes following application, and subsequent absorption occurs from residues in some state of dehydration, presenting unusual problems in resolving mechanisms of absorption. Since ion exchange may play a role in the uptake of certain nutrients, e.g., potassium, precautions should be taken in studies employing radioisotopes. The absence of isotopic exchange must be established where the presence of labeled nutrients in the leaf is used as an index of net uptake. If external and internal factors regulating absorption are to be elucidated, a control of these variables is essential, and an accurate means of differentiating between absorption and subsequent transport must be developed.

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LEAF PROTEINS[1]

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Various motives prompt a study of leaf proteins. They are the most important source of protein for domestic animals and could be an important source for man. They are the starting material from which the proteins of seeds and tubers are, for the most part, derived. They are intimately involved in the processes of virus multiplication and possibly in some of the mechanisms protecting the leaf from various forms of infection. Finally, since much or even most of the protein of the leaf is made up of enzymes, variation in their amount and nature will accompany physiological changes in the leaf whether these are the consequence of age, adversity, or changes in nutrition and environment.

These diverse motives lead to equally diverse experimental methods. If the leaf is a source of fodder there is little need to fractionate it; yield, composition and digestibility are the important qualities. Fractionation is necessary before leaves can be used as a bulk food for man or before the individual enzymes or enzyme complexes can be studied. When fractionation starts, the study of physiology ends and is replaced by the study of artefacts. This can be useful, as long as it is clearly recognised, for it gives evidence about the minimum complexity necessary for an action. But a study of the normal processes that are going on in a system is more useful practically and it is much more difficult to manage. For success, the sceptical outlook is needed of a detective on the lookout for pickpockets or of a spectator trying to see how a conjuring trick is done. The most significant changes in the whole process may be going on during an apparently innocent and insignificant phase of the operation. Recognition of this difficulty must not become an obsession, as perhaps it did with J. S. Haldane who latterly became unwilling to use any result that did not come from the normal excreta or exhalations of an unmodified animal, but it should stimulate caution. Information derived from leaves that have been detached, rubbed, infiltrated, sliced, cooled, dried, chopped or "homogenized," is often highly relevant to physiological occurrences in the plant. But the relevance must be demonstrated rather than assumed.

Studies on the Whole Leaf

There is an abundant literature on the effect of fertiliser treatment on the nitrogen content of the leaf; it shows that variation by a factor of four is not unusual in differently treated leaves of the same species and age. It is this variation that makes the direct use of leaves in quantity as human food hazardous because when the ratio of protein to fibre falls below a certain

[1] The following abbreviations will be used: ATP (adenosine triphosphate); DNA (deoxyribosenucleic acid); RNA (ribosenucleic acid); TMV (tobacco mosaic virus).

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value, which has not been measured and probably varies with the type of leaf, increased peristalsis would make the leaf useless as a protein source. A few examples of leaves rich enough in protein to be useful as bulk foods have been given (106) and a plea was made for more research on how to maintain a regular high value. In most of this work, nitrogen rather than protein was measured but, unless leaves are harvested soon after fertilisation or high-nitrogen shade leaves are used, at least three-quarters of the leaf nitrogen is generally protein nitrogen. The conclusions that have been drawn about the amino acid content of whole leaves will be discussed later.

Two methods are available for getting more detailed information about the distribution of protein in the living intact leaf: microscopic recognition of characteristically shaped bodies *in vivo* followed by identification by staining and microspectrometry based on the assumption that absorption at 280 mμ is characteristic of proteins (28). These methods have not so far given much information about normal leaf proteins, but crystals that contain virus can be recognised in infected leaf cells (57, 149) and even less well defined bodies in other cells. Identification becomes more certain when the tissue is fixed and stained; however, these processes bring with them the probability of migration of material during the fixation, and histochemistry is notoriously unspecific (69, 107). By these methods, crystals that appear to contain protein have been recognized in many species; recent additions to the group are *Albuca festigiata* and *Opuntia inermis* (84, 144). Electron microscopy has given information about the distribution of virus particles in infected leaves and also about organized structures, that are probably protein, in normal *Gingko biloba* (15). It is difficult, by these techniques, to turn the probability into a certainty and to be sure that what is being seen is protein, but observations on unfractionated material are an essential first step in localising proteins in the cell.

The assumption that where an enzyme activity is found a protein is present, meets with general acceptance, and so does the assumption that N insoluble in boiling water and organic solvents is protein. The second assumption is probably the less valid of the two because N-containing lignins have been postulated, and glucosamine is a recognised plant component (43) though it has not so far been recognised as part of a molecule large enough to be confused with protein. All the enzymes so far investigated have contained protein so that changes in the metabolic behaviour of a tissue reflect changes in the protein in it and the histochemical recognition of an enzyme may legitimately be accepted as a mode of localizing protein. This technique has been applied much more assiduously to roots and seeds than to leaves and it need not be discussed here because there is a recent, admirably critical review (35).

The Methods of Disintegration

Very little of the protein of the leaf can be studied without disintegrating the tissue. Some is present in the fluid exuded when hydrostatic pressure is applied to the petiole (55) but little work has been done on it. The proteins

in latex have been much more extensively studied because of their association with rubber and their importance as proteolytic enzymes. They have, however, been discussed recently (105) and the latex vessels pervade the whole plant so that latex protein is not peculiar to the leaf. The insectivorous plants secrete proteases but these do not seem to have been investigated recently.

There are no general rules governing the preparation of extracts from leaves (104). The problems of both extraction and subsequent fractionation can sometimes be simplified by using selective methods; thus, the juice that runs from frozen and thawed, but unminced, barley leaves contains carboxylase (13) and extracts from carefully dried leaves contain α-hydroxy acid oxidase (64). These methods are selective because much of the leaf protein coagulates on freezing or drying; they are therefore not useful when the more sensitive proteins or structures are being investigated. The various possible methods of disintegrating a tissue by rubbing or cutting it in dilute suspension or without added fluid were compared recently (104). Two convenient units for disintegrating leaves quickly under controlled conditions have been described since (33, 108). An attempt has also been made to analyse the probable range of particle size in the heterogeneous mixtures made by the devices so inaptly called "homogenisers" (2); the conclusion is that for disintegration in the annulus between concentric cylinders, it is preferable to have reciprocating movement parallel to the axis rather than rotation about the axis. Enzyme activity is associated with such fragile particles, and disintegration is so likely to alter the state of proteins, that it is a pity the different methods of disintegration are so seldom compared on the same starting material and with the same final treatment of it. It is only when we know how much the properties of the final preparation depend on the details of the preparative method that conclusions can be drawn about the possible behaviour of the system *in vivo*.

Mechanical damage is the most obvious hazard to which an intracellular structure is exposed, but in many tissues the subcellular particles have considerable mechanical strength. The greatest hazard then is that, during grinding, substances are exposed to an environment different from that surrounding them normally. Kitchen experience shows that many cut or bruised surfaces undergo rapid changes on exposure to air, and devices (32, 118), have been described for grinding in the absence of air. This principle has now been used (18) to facilitate the preparation of colourless leaf protein fractions. It is preferable to exclude air rather than to try to inhibit oxidations by the addition of enzyme poisons, which may be difficult to remove afterwards, or reducing agents, which may produce powerful oxidants as they themselves become oxidised.

The various components of the leaf may interact detrimentally on one another when they come into contact after grinding. Acid vacuolar contents can be neutralised by infiltration with ammonia (19, 66), or by grinding in the presence of a suitable amount of $Mg(OH)_2$ (111) or in large volumes of buffer. It is difficult, by these means, to maintain a stable pH greater than

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6.5 in mushes from the leaves of many species because of the action of pectin esterase (50); conversely, other species become alkaline on grinding (51) and this also can bring about changes in the proteins. The most serious interference comes from tannins which are well known protein precipitants. Tannins in the leaf have been held responsible for the difficulty encountered in making infective extracts from some virus-infected plants (4, 138). This effect can be minimised, either by extracting the tannins from dried leaf with nonaqueous solvents before extracting the virus (140), or preferably, by adding substances to the extracting fluid that combine with the tannin and so protect the viruses. Nicotine has been most widely used for this purpose (73, 137, 138).

It may be necessary to take care in interpreting these results because nicotine is said (41) to inactivate TMV in crude sap though, at the concentration used, it does not inactivate purified virus (5). More recently the effect of leaf tannins has been countered by adding polyglycols ("Carbowax") to the fluid in which the leaf is extracted. In a review in this journal, on the application of this technique to the preparation of active chloroplasts, Clendenning made the interesting points: (*a*) that part of the effect may not be so much to remove tannins from the environment as to precipitate them as a protective, stabilising coat on the chloroplasts and (*b*) that our choice of certain leaves as foodstuffs and as good chloroplast sources may depend on the same aspects of their chemical composition. Similar factors probably also control susceptibility to virus infection.

Bulk Preparations of Protein

Whether we are concerned with forage for ruminants, with more carefully selected leaf material to add to pig and chicken feed, or with still more carefully selected leaves for human food, the amino acid composition of the bulk protein is important. Considerable effort has, therefore, been put into amino acid determinations on digests of whole leaves and variations have been claimed (117, 131). Although these results are plausible, some of them do not seem to fall outside the possible uncertainties introduced by hydrolysis in the presence of varying amounts of carbohydrate and other leaf components. In view of this uncertainty it is a pity that so few of the analyses are expressed in a manner that makes it possible to find out what proportion of the protein nitrogen is being accounted for. This is a point that has been made more fully elsewhere (110). It must be stressed, however, because the significance of an apparent difference in amino acid composition clearly depends on what we know of the manner in which losses fall on the different amino acids when the nitrogen unaccounted for is a significant part of the whole. It may well be that what varies is the extent to which analysis is thwarted rather than the concentration of any one amino acid. Results can be made more trustworthy by hydrolysing very dilute suspensions of leaf but it is preferable to use extracted proteins for the analysis. This has only rarely been done [e.g. (128, 150)]; when it is, the trustworthiness of the amino acid analyses is high but the extent to which the analysis gives information about

the whole protein of the leaf is uncertain. The position is clearly difficult; we can have trustworthy analyses on uncertain starting material or uncertain analyses on a definite starting material. Bryant & Fowden (12) adopted an intermediate course and extracted 90 per cent of the protein of daffodil leaves along with so much other material that the concentrates used for analysis contained 8 to 13 per cent N. Bulb scale leaves and the white basal parts of the leaves had different compositions from the rest. The green parts of foliage leaves from plants at the flowering stage contained less serine, and more threonine, amide-N, and possibly leucine, valine, and methionine than from plants before flowering. There were no significant differences between the different green parts of the same leaf although the apex is considerably older than the lower parts of the leaf. Another approach is to compare analyses on the whole leaf, the residue after extraction, and the extracted protein fractions. A partial attempt at this (60), which did not include analyses on the residue, gave higher values in the extracted protein than in the original leaf for all the amino acids determined, which strongly suggests that the differences were due to analytical failures.

There is clearly a great need for improved methods of protein extraction. Existing methods have been discussed elsewhere (105); they are essentially those used by Rouelle in 1773. More attention is now paid to salt concentration and pH with the result that it is easy to extract 60 per cent of the protein from soft fresh leaves and it is possible to extract 90 per cent. Extraction gets worse at the leaves mature or wither and it may be negligible from harsh textured leaves or leaves rich in tannin (17). There are scattered comments in the literature on the use of very alkaline solutions, of aqueous solutions of the powerful solvents such as urea, salicylate, thiocyanates, etc., and of nonaqueous solvents such as phenol, formamide and formic acid. I have not found these sophisticated solvents any more satisfactory than water on fresh leaves and the experience of others seems to be the same. A general method for extracting leaf proteins would, however, be so useful that the search is worth continuing although an effective agent may well dissolve the whole leaf and so substitute an intractable solution for an intractable solid.

The bulk protein of the leaf is important because it is a potential source of human food that could be used more generously than would be advisable with even high quality but unfractionated leaves. This use was suggested in 1926 (34) but large scale machinery suitable for extracting the protein has only recently been made. The two most successful processes are to pulp the leaves in one machine and immediately press in another (102, 112, 116) or to use a modified screw expeller (115). The former arrangement seems preferable. Existing machinery is able to handle suitable leaves at rates up to 2 tons an hour and liberate 50 to 60 per cent of the protein in a form that can be separated from the juice by heat coagulation or precipitation with acid. The crude product made in this way contains 10 to 12 per cent N, 5 to 12 per cent lipide and 5 to 12 per cent starch.

The advantages claimed for leaf protein production as a supplement to other forms of agriculture are that the yields of protein per year per acre

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could be higher than with other protein-rich crops and very much higher than those attainable if a crop is subjected to animal conversion. These are real advantages provided production is not too expensive and provided the nutritive value is as high as the amino acid composition suggests. Conflicting guesses at the costs of production have been made (99, 116), and conflicting opinions about the probable usefulness of leaf protein in practice depend on them; but no one has tried to determine the costs experimentally. Pig feeding trials on carefully made protein show that it is as good as or a little better than fish meal (3a); the great variability of results with crude batches of protein (24, 25, 116) suggests that this protein is more easily damaged in processing than the more familiar seed proteins. There is clearly need for much more research. The justification for considering these points in the context of plant physiology is that, if it is agreed that one of the probable means for satisfying the world's increasing need for protein is the large scale manufacture of hitherto unused materials such as leaf protein (113), much plant physiological research will be called for. Cultural conditions for ensuring exuberant production of high-protein leaf will have to be found and it may well be that a completely new group of domesticated plants will be the vehicle for this production.

Much effort has been put into the mass cultivation of algae as an unconventional source of protein for human food (134) but it is important to remember that no more is known about the cost and nutritive value of that protein than of leaf protein. The problems involved in making algal protein and leaf protein are essentially similar. Their amino acid compositions are also similar (37) but it does not seem to be so easy to make a concentrate from algae with as high a protein content as that made from leaves. In some environments, notably those with an acute shortage of space and water, it is probable that algae would be more productive than higher plants, but the yield per acre is as great with higher plants as with algae if conditions are comparable (136, 143). The contrary claim, though commonly made (134), can only be supported if algae grown under exceptionally pampered and expensive conditions are compared with farm crops exposed to the normal rigours of large scale husbandry.

Studies on Isolated Components of the Leaf

Plants have a general tendency to use carbohydrates in the structural roles for which animals use proteins; we would not therefore expect to find much protein in their cell walls and protein is hardly mentioned in a recent review (90). The argument has already been developed (105) that the similarity of the ratio of N to chlorophyll in the chloroplast fraction of tobacco leaves, to that in the fibre residue after extraction, bears out expectation and justifies the view that most of the N in the fibre comes from entrained chloroplasts. From the meagre literature on cell wall proteins two papers may be cited, each concerned with the stem rather than the leaf; there is good evidence that cell wall protein is involved in the leaf movements of *Mimosa* (147) and that the action of indoleacetic acid depends, at least in part, on its

influence on the fixation and release of pectin methylesterase by the cell wall (44).

Chloroplasts.—Protein, in a vigorously growing leaf, is predominantly located in chloroplasts. These are a clearly definable type of subcellular particle. The remainder of the protein is in a heterogeneous group of particles large enough to be resolved in the microscope and sedimented on normally available centrifuges (nuclei, mitochondria, microsomes, etc.), in cell walls, and in the so-called "soluble protein." The details of the technique used to fractionate a leaf extract control the precise position in this system occupied by many distinguishable proteins, and the categories merge into one another because of the indefiniteness of the criteria used for even histological recognition, let alone recognition after the damage inseparable from extraction.

There is now a vast literature on the internal structure of chloroplasts. This is not the place to review it but it is clear that the structure is different in different species and that most chloroplasts contain up to 100 grana embedded in a stroma. Microscopy suggests that the lipides, including chlorophyll, are concentrated in, even if not confined to, the grana but protein is probably more widely distributed throughout the chloroplast. Preparations have been made by fractionating dispersed chloroplasts and described as preparations of grana, or of some of the other components, but it is by no means clear that the claims are fully justified and that complexes with other material have not been formed. The assumption is not valid that those preparations that have the greatest ability to bring about some enzyme action are in the least modified state. Stability, and so enhanced activity, are often a result of complex formation. Thus the photosynthetic activity of intact chloroplasts is enhanced if substances such as sucrose, methanol, glycols and the "carbowaxes" are added during the preparation; with the carbowaxes at least, this enhancement may be a consequence of precipitation of tannins and other components on to the outside of the chloroplast (17). The accreted layer is presumed to stabilise parts of the Hill reaction system.

The absorption spectrum of a chlorophyll solution differs from that of a suspension of chloroplasts. This difference has been taken for many years as evidence that the chlorophylls exist *in vivo* as complexes and several preparations containing protein have been described as the native holochrome. Some doubt has been thrown on the basic assumption by studies of the shift in position of absorption bands brought about by scattering, when a pigment is present as particles with dimensions of the same order of size as the wavelength of light (71). But better evidence for the reality of the protein complex has been gained by the isolation from etiolated leaves of protochlorophyll (45, 68, 92, 129). This protein complex presumably comes from the plastids and it is converted by light into a chlorophyll:protein complex with the usual colour. This transformation takes place in dialysed solutions so that components of low molecular weight are probably not involved in it. Protochlorophyll appears to be homogeneous on the ultracentrifuge ($S=16$); this fact, taken in conjunction with the ability to undergo a specific action similar to that which it presumably undergoes *in vivo*, makes it unlikely that the

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complex is an artefact. The status of the crystalline chlorophyll:lipoprotein complex that can be made from picoline extracts of chloroplasts is still uncertain (89).

The protein that can be dissolved by phosphate solutions at pH 7 from chloroplasts that have been extracted with cold acetone, will combine with chlorophyll to give a photosynthetically active complex (142). This is not only an observation of great intrinsic interest but it brings out the need for caution. If complexes can be made *in vitro* it is unwise to assume that it is only the normal, specific ones that are being made.

Extracts containing the protochlorophyll:protein complex were made from etiolated bean leaves ground at pH 9.6 without preliminary separation of the chloroplasts. The original location of the complex is therefore somewhat uncertain. A component from the expanded green leaves of several species has the sedimentation constant S = 16 to 19, and is commonly referred to as "Fraction I Protein." It is generally assumed that this is a cytoplasmic component, but Lyttleton & Ts'o (76) question the assumption because of the relative constancy of the ratio of "Fraction I" to chlorophyll. The yield of "Fraction I" diminished if the chloroplasts were aged or damaged during preparation which suggests that this is not a case of secondary absorption during the isolation.

There is also uncertainty about the presence of nucleoproteins in chloroplasts. Holden (52) extracted 38 per cent of the P from a crude preparation of chloroplasts with perchloric acid in a manner characteristic of RNA but she found that, after incubation, the amount of RNA was greatly diminished presumably because of hydrolysis by leaf ribonuclease. In more carefully fractionated chloroplast preparations Jagendorf & Wildman (53) found very much less RNA. Amounts similar to those found by Holden have however been found by others (16, 20, 76, 91) and two nucleoproteins have been characterised to some extent by electrophoresis and differential salting out (83, 126). There is some conflict, but no more than must be expected when leaves of different species and ages are being used, when they are fractionated at different, and often unstated, intervals after harvesting, and when varying precautions are taken to work quickly and keep the extracts cold during the fractionation.

Isolated chloroplasts will carry out most of the processes of photosynthesis so that it is reasonable to conclude that the enzymes are present which are needed to bring about the intricate series of reactions dealt with elsewhere in this Annual Review. Several enzymes have been investigated without clear evidence being adduced that they occur in or on the chloroplasts; the difficulties arising in establishing this have been discussed elsewhere (105). The most fully characterized enzyme is probably triphosphopyridine nucleotide diaphorase which contains flavine-adenine-dinucleotide. The enzyme is centrifugally homogeneous (S = 3.5) but the best preparations still contain two electrophoretic components (3). It is probable, for physiological reasons, that the carboxylation enzyme, which makes two molecules of phosphoglyceric acid from ribulose diphosphate and CO_2, is present in chloroplasts

though this has not been unequivocally demonstrated (148). Purified preparations are ultracentrifugally homogeneous with S = 17, but this is such a usual value for proteins from the leaf that it has little bearing on the purity of the preparation. The cytochromes, especially *f* and b_6, are present in chloroplasts (49); their properties were reviewed here last year (130).

Mitochondria.—The microscope shows what a normal chloroplast looks like in the cell and it is only during attempts to explore its internal structure that the artefact-engendering processes of fixation and fractionation are called in. The normal mitochondrion is more elusive and its study probably awaits full exploitation of the extra resolution that is possible with reflexion microscopy. Mitochondria are probably pleomorphic *in vivo* (46, 47, 88); they are also plastic and fragile so that the forms finally seen are the result of both the breaking of elongated forms and the rounding out of forms with initially more complex shapes. All this makes evidence for the presence or absence of enzyme systems in mitochondrial preparations, after they have been exposed to repeated centrifuging, difficult to assess. These changes in shape have sometimes been interpreted as osmotic effects on an enveloping membrane and this is undoubtedly a possible explanation, but it is not the only one. Essentially, a membrane is a structure that can be peeled off and recognised in isolation; a structure like that has not been found in mitochondria. It is just as likely that the surface properties are those of interfaces, or even of a sponge-like structure, in which the mutual disposition of the parts of the sponge depends on the electrical and other properties of the surfaces and the fluids between them. If the mitochondrion has such a structure, it would offer great opportunities for the acquisition and loss of enzymes so that all statements made so far about their presence or absence would have to be accepted with reserve. Enzymes are, however, probably more often lost than gained during purification; with many enzymes, hexokinase for example (122), the proportion of the total enzyme activity that is found associated with the mitochondrion is increased by careful preparation.

Animal mitochondria have been more thoroughly investigated than plant mitochondria because academic research on animals is more generously endowed [e.g. (123)]. Roots or etiolated shoots are the preferred plant material because preparations made by simple differential centrifugation are then only slightly coloured. The leaf has therefore had relatively little attention. It is disconcerting, though no doubt salutary, to have the presence of components other than mitochondria blatantly revealed by the bright green colour of the preparation; colourless contaminants are less obtrusive. The chloroplasts in most leaves are so fragile that some get broken by any grinding technique that is vigorous enough to liberate the mitochondria. The problem therefore is not to remove chloroplasts 5–10 μ in diameter from among 1 μ mitochondria but to remove their 1 μ fragments. James & Das (54) got electrophoretic separation but considered the technique impractical. By centrifuging in a density gradient they separated chloroplasts uncontaminated by mitochondria and showed that colourless or pale yellow mitochondrial preparations could be made but did not study them. This seems to

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be a problem to which Albertsson's (1) technique of separating particles according to their differential affinity for two immiscible liquids would be well adapted if liquids that did not damage the mitochondrial integration can be found.

Mitochondria account for 4 to 5 per cent of the protein of tobacco leaves (77, 97). This is only a tenth of the amount of protein present in the chloroplasts but the distribution of N among the various leaf fractions has not been measured consistently enough to show whether it is a representative ratio. It is in good agreement with the usual histological appearance, though leaf cells vary greatly in the apparent number of mitochondria in them; there are, for example, very few if any in the leaf palisade cells of *Vicia faba* (54).

Work on the enzymes of mitochondria has recently been reviewed (70). Most of the enzymes involved in the tricarboxylic acid cycle have been found in preparations from a range of species including the gymnosperms and pteridophytes (38); the activity varies and Sisakyan *et al.* (125) suggest that it is favoured by conditions, such as shade and low temperature, that limit photosynthesis. Pierpoint (97) used several different extraction fluids in making preparations from tobacco leaves and found that, although the preparations appeared to be similar and all contained about two-thirds of their N in the form of chloroplasts, their enzymic activity varied greatly. The standard extracting fluid contained phosphate, sucrose, ethylenediamine tetra-acetic acid, 2-amino-2-hydroxymethylpropane-1:3-diol (TRIS), and citrate; these components are listed in order of increasing importance for preserving succinoxidase. Phosphate was unimportant and citrate vital. The polyglycols, heparin and dextran, which have been used in place of sucrose in other systems, aggregated the preparations into an easily sedimented mass. The leaves of young plants and the younger leaves from old plants gave the most active preparations. It is likely that measurements of this sort could be used to give substance to the elusive concept of physiological age.

The enzymes discussed above have not been extracted from leaf mitochondria though they have from pea seedling mitochondria broken up by shaking (27). Other activities have seldom been looked for. However, a crude mitochondrial fraction from French bean leaves produces a substance that inactivates TMV (11) and, under suitable conditions, tobacco leaf mitochondria inactivate the infective fragments of TMV (114).

Nuclei are a characteristic feature of the histology of leaf cells but hardly anything is known about their proteins. In the normal systems of fractionation, nuclei and their fragments are probably distributed between the mitochondria and the microsomes; it is often assumed that the extent of this contamination can be gauged from the amount of DNA in the preparation but this is an assumption that is plausible rather than necessary (107).

Microsomes and Viruses.—Some plant viruses, notably TMV, are so stable that it is easy to separate them from components characteristic of normal leaves. The processes of separation do not leave the virus unaltered but it remains infective and that, for many purposes, is sufficient (109). When the less stable viruses are made by differential ultracentrifugation, contam-

ination by leaf microsomes is probable because they have sedimentation constants in the range (60 to 200S) that includes most plant viruses. This prompted a study of the microsome fraction from young tobacco leaves (101). The yield can be as high as 5 gm. per kg. of leaf but 1–2 gm. is more usual; this fraction contains about a tenth of the protein of the leaf and it contains most of the RNA. In a few weeks at 0°, and more rapidly on incubation or on repeated ultracentrifugation (111), part of the protein denatures and precipitates. Similar material has been prepared from young leaves of several other species (22, 23, 75, 101, 111) and from pea seedlings (139) but those made from beet petioles contained little or no nucleic acid (80). It seems probable that this macromolecular nucleoprotein is a genuine component of young leaves; there is no good reason to think that in young leaves, the material is associated with the chloroplasts. Such an association is probable in older spinach leaves (76). It is also probable that much of the material that finally appears in a microsome preparation is derived from the breakup of laminar or filamentous structures in the cell. The position is by no means clear. From different starting materials, and using different methods of fractionation, nucleoproteins of much lower molecular weight can be made; these could aggregate under other conditions to make the material in the microsome fraction. On the other hand macromolecular nucleic acids have been found in plants (74) and located in chloroplasts (72); these could combine with protein during extraction. Confusion will remain until the same preparative methods are applied to leaves of the species and ages that appear to be giving dissimilar results and until all the different preparative methods have been applied to aliquots of the same batch of leaves.

Microsomes carry a sufficiently large proportion of the ribonuclease of leaf sap to account for their own instability *in vitro* and the instability is increased by adding more enzyme and in conditions that favour the action of the enzyme. Microsomes have other more interesting activities; thus, they can incorporate labelled amino acids *in vitro* (23), catalyse interchanges between phosphoric esters, and also incorporate nucleotides (145, 146). The last two activities, unlike amino acid incorporation, are not dependent on the integrity of the particle but can be brought about by its fragments. Amino acid incorporation by microsomes may be an early step in protein synthesis because this fraction of a leaf picks up most of the labelled amino acid on brief exposure *in vivo* (132) but, as the authors quoted realise, the uptake of a labelled amino acid is not the same thing as protein synthesis. It may be a replacement of an unlabelled amino acid by a labelled one or it may be an example of the unspecific combination that Cornwell & Luck have described (21).

The plant viruses are still the largest and best characterised group of leaf proteins; their properties cannot be discussed in detail here but a comment on some generalities is appropriate. There has recently been some salutary conceptual spring-cleaning and several irrelevant and improbable assumptions have been discarded. Thus, in spite of observation (6) and argument (7, 9, 98), many had clung to the idea that infection was a simple process in

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which an extraneous substance, in a susceptible cell, engineered the production of identical copies of itself and nothing else. There were even those who thought the process could be made still simpler by assuming it to be "autocatalytic" and the word "self-duplication" was much used. It is now generally agreed that infection is a complex aberration of the synthetic processes of the cell which produces, among other things, copies of the infecting agent. There is no information about the number of steps involved in this response to an invasion and it is only when the number of steps is of the order of 1 that the concept of autocatalysis is useful. To use the phraseology of formal logic—an infective agent is the necessary but not sufficient cause of its own multiplication. There is no need to discuss the properties of the TMV companions here for they have been surveyed recently (10, 110).

Another demolished simplification is the generalisation that plant viruses are ribonucleoproteins and animal viruses deoxyribonucleoproteins. It was obvious that the sample from which this generalisation was drawn was inadequate (100); but logic, rightly, was less effective than experiment, and the generalisation was overthrown by the gradual demonstration that most statements made in the 1940's about the nucleic acids of animal viruses were wrong. Many appear to contain RNA. So far the presence of RNA has not been shown to be necessary for the infectivity of a bacterial virus nor of DNA for the infectivity of a plant virus. But the group of viruses studied is still nearly as restricted as in 1946 so that no metaphysical implications should yet be drawn from these apparent exclusions. It is also important to remember that the nucleoproteins are the minimum rather than the normal vehicles of infection and that, if the conditions of infection are suitably controlled, even simpler fragments can be infective. Markham's observation that there is firmly bound spermidine in TMV and turnip yellow mosaic virus (78) introduces us to a very interesting new component of some viruses.

The Smaller Proteins.—Little is known about the original location of most of the proteins with particles too small to be visible with the microscope, because the first steps in the isolation may bring about, and may be intended to bring about, the disruption of links with larger particles. However they originate, these proteins can be recognised in three main ways: (*a*) by measurments that, with varying degrees of specificity, depend on the presence of material satisfying physical or chemical criteria for protein; (*b*) by measurements of the intensity of an enzyme action, whether it is the one brought about by the enzyme *in vivo* or a surrogate for it; (*c*) by following a more or less accidental but specific property of the protein such as its ability to be an antigen, toxin or haemolysin.

The first method has been extensively used to follow the effects of virus infection on the amounts of the different components that can be recognised in leaf extracts stabilised so as to give satisfactory electrophoretic and ultracentrifugal patterns. The processes of stabilisation are, in essence, processes for removing microsomes, mitochondria and other large particles. Bawden & Kleczkowski (4a) have recently discussed the interpretation of these meas-

urements and they stress the differences that exist between uninfected leaves in different positions on a tobacco plant. There has been only one careful study of the changes in the sedimentation pattern during the development of a leaf and in it the proteins were divided into two broad categories according to whether their sedimentation constants were about 4 or about 19. Although each category is obviously heterogeneous, certain regularities are apparent (30). During the phase of rapid leaf growth the amount of each type of protein in a leaf increased exponentially but proteins of the type S = 19 increased more rapidly so that, from being less than a fifth of the total in leaves of the Maryland variety of tobacco only 2 cm. long, they made up half the total 10 days later when the length was 20 cm. During the phase of senescence these proteins (S = 19) also disappeared more rapidly than the others.

Measurements depending on the activity of an enzyme are seriously confused by the widespread occurrence of latent enzymes. It is possible therefore that, when an enzyme activity appears to increase as the result of a physiological change, there has been no actual synthesis of new protein but rather a change in the configuration, combination, or disposition of a pre-existing protein. The latency of enzymes presumably has a regulative function *in vivo* and examples of it, mainly in microorganisms and animals, have been usefully surveyed (133). As is well known, extracts of broad bean blacken on exposure to air because of the action of phenolase. Substances able to act in this way are associated with the pellets when bean leaf extracts are ultracentrifuged so that preparations of viruses and other macromolecular leaf components blacken on storage. However, most of the phenolase enzyme appears in a latent state in the low molecular weight fraction. It can be activated a hundredfold by repeated precipitation with ammonium sulfate, incubation alone, brief exposure to pH's around 3 or 11 and to anionic detergents (61, 62). Kenten gives reasons for thinking that the activation is not a consequence of the removal of an inhibitor from the environment but rather of the irreversible fission of a complex between the active enzyme and a protein that covers sites necessary for activity. The polyphenolase activity of acetone precipitates from tobacco sap can be increased by heating the dry material at 100°C. but here the opposite interpretation, that interfering enzymes are being removed, is favoured (48). Ether and some other solvents activate the phosphatidase of chloroplasts (58) and cytoplasm (26) and the adenosine triphosphatase of pea mitochondria is activated when the mitochondria are exposed to certain damaging conditions (36).

In the early days of immunology, plant proteins were extensively studied; their serological activity has aroused less interest recently. This is partly because the proteins in many leaves, tobacco for example, are relatively poor antigens. This makes possible the use of incompletely purified virus preparations as antigens when diagnostic antiviral sera are being made, but the antigenicity of the normal proteins is sometimes great enough to make it necessary to precipitate the antibodies that react with normal proteins by a preliminary absorption of the serum with an extract from uninfected leaves

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(127). The techniques and results of serological testing for virus infection have recently been thoroughly surveyed (82, 127). It is by no means clear why normal leaf proteins tend to be poor antigens, but it may be that the animal that is being immunised is able to get rid of these proteins more easily than of some others by hydrolysis or in some other way (103). Many leaf extracts precipitate unspecifically with normal sera; this form of interference can be minimised by preliminary fractionation of the serum (119). The existence of a common antigenic specificity associated with the proteins that have sedimentation constants about S = 19 in a wide range of plant species (31) introduces another element of uncertainty in the use of serological reactions for the specific recognition of leaf proteins.

It is not possible to discuss all the leaf proteins that have been characterised to some extent and every reviewer would make a different arbitrary choice. Three proteins have been purified sufficiently far for the preparations to crystallise: catalase from spinach (42), glyoxylic acid reductase from tobacco (151), and glycollic acid oxidase from spinach (39). The methods used for these purifications suggest that more difficulties are encountered here than in making purified enzyme preparations from such starting materials as pancreas; these difficulties probably arise because the leaf is able to carry out an immense number of actions so that each enzyme in a leaf extract makes up a very small proportion of the total. Some enzymes, to which one name is given, are themselves heterogeneous. Thus there are at least two phosphoesterases in peas, separable by chromatography but not differentiable by comparisons of the ratios of their activities towards substrates as diverse as glycerophosphate, polyphosphates and metaphosphates. Reasons are given for thinking that this heterogeneity is not produced during the processes of purification (95, 96). A study of the electrophoretic behaviour of the dipeptidases in cabbage juice led to a somewhat different conclusion (29) for, although the fractions showed differing activities towards different substrates, it seemed possible that these apparently different enzymes were artefacts made from one original enzyme.

Two other enzymes deserve mention because of the general interest of the actions they catalyse, although purification has not yet been carried very far. Ribonuclease has been freed from unspecific phosphatases that act on ATP, metaphosphate, etc. (94). This enzyme, like the pancreatic enzyme, is able to build up polynucleotides if supplied with suitable substrates but the pancreatic enzyme is a phosphodiesterase attacking esters of pyrimidine nucleotide 3′ phosphates whereas the leaf enzyme attacks all the internucleotide links giving at first nucleoside 2′3′ phosphates and later completing the hydrolysis of the purine cyclic phosphates to give 3′ phosphates (79). Pancreatic ribonuclease is now so widely used as a tool for investigating, *in vivo*, actions thought to depend on RNA that it would be well if parallel studies were made with the leaf enzyme; this is particularly true when an action in a plant is being studied.

The toxicity of bracken for many animals is in part due to the presence of thiaminase in it. There are some animal and microbial sources of the enzyme

and it is present in some other ferns, but it is only known to occur in one higher plant, *Celosia crista* (40). Stable preparations of the enzyme have been made from bracken and they catalyse the substitution of a variety of bases for the pyrimidine moiety of thiamine (63). Preliminary teleological thinking suggests that it is rather improbable that this reaction is important *in vivo*, even making allowance for the erratic distribution of the enzyme. It seems probable that the enzyme has a different role but that, in certain species, its specificity is sufficiently elastic to include the action on thiamine. One possibility is that it is concerned with the transfer of groups on to and from the nitrogenous heterocycles of nucleic acids. Some purines and pyrimidines take part in the transfer reaction with thiamine (63), and thiaminase in the presence of thiamine, inactivates the infective nucleic acid fraction from TMV (114). These reactions are compatible with the idea that the enzyme is concerned normally with reactions involving nucleic acids though obviously they are not positive evidence for it.

Effects of Age and Cultural Conditions on the Amount and Composition of Leaf Proteins

The plasticity of plants and the readiness with which the environment affects their biological responses are a constant cause of trouble to those who use leaves as a source of enzymes or for assay. This plasticity probably accounts for many published contradictions and aspects of it have been commented on in an earlier review (105). Even if care is taken to use the same variety of plant in different laboratories, the usual temperature, illumination, humidity, nutritional status, and age at harvest may well be different. The last is especially important. It is now generally recognised that the percentage of protein in a leaf decreases as the leaf matures. This is mainly because protein synthesis does not keep pace with carbohydrate synthesis; it is only in late stages of development that there is a net loss of protein from the leaf. In a deciduous tree this no doubt affects all leaves equally but Tanaka (135) finds that this is not so in rice; a larger proportion of the protein in later-formed leaves was translocated to the grain. Differences in the amino acid composition of young and old leaves have often been claimed but, as has been said earlier, it is doubtful whether the analytical methods are adequate to establish the differences. The analyses have seldom been done on protein fractions separated from the leaf fibre in the same way. The claim (124) that cystine and threonine increase with age at the expense of serine, valine, tyrosine, leucine, isoleucine, and lysine is, however, based on analyses of isolated chloroplast proteins.

The increase in the ratio of protein with sedimentation constant $S = 19$ to protein with $S = 4$ as tobacco ages has been commented on (30) as have the differences in the electrophoretic patterns of leaves in different positions (4a); one peak was found only in old or N-deficient plants and another was found in upper leaves but only rarely in the bottom two or three. In young barley, 95 per cent of the cytochrome oxidase is attached to the chloroplasts but with age it appears increasingly in the cytoplasm (121). The hydrolytic

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enzymes increase as tree leaves mature (59) as does polyphenoloxidase in cereals but peroxidase appears early and then declines (120). If this is a general phenomenon, it could explain the relatively high peroxidase levels found in dwarf varieties of tobacco, beans and maize (56). There is more catalase in the upper than the lower leaves of some trees but the difference is less marked in drooping varieties; it is suggested that the smaller difference induces the drooping habit (85). Not only do leaves in different positions on a plant have different compositions but there are also differences, in pH for example, between different parts of the same leaf.

Vickery (141) has carried this type of experiment a stage further by examining the ability of different leaves to recover from stress. He concludes that this is correlated with the protein content of the leaf and that in low protein leaves the enzymes necessary for recovery have been depleted.

Much has been written on the effects of macronutrients on the protein content of leaves but little on the consequent changes in the character of the proteins in them. The interesting observation has been made recently, however, that sulfur deficiency increases the electrophoretic heterogeneity of the proteins in lucerne leaf cytoplasm (81).

Many of the micronutrients are components of enzymes so that it is reasonable to expect certain enzymes to be diminished in plants that are deficient in certain elements. This has been observed for several oxidizing enzymes in tomatoes (87) and for nitrate reductase in molybdenum-deficient cauliflowers (14). An unexpected effect of deficiency is the formation of an excess of apparently unrelated enzymes. Thus, zinc deficiency leads to peroxidase excess (87); copper deficiency to isocitric dehydrogenase excess (86), and boron deficiency to tyrosinase excess (67). Each of these enzymes makes up, of course, only a tiny fraction of the whole leaf protein, but these nutrient variations seem to affect so many enzymes that they may well bring about significant final changes in composition. It is however, not clear to what extent a metal-free enzyme precursor, presumably with the amino acid composition of the enzyme, is present in those plants in which an enzyme is deficient; nor is it certain that some of the effects observed are not a consequence of varying degrees of activation of latent enzymes.

Finally there are the effects of virus and other infections. It is reasonable to suppose that most of the symptoms by which infections are finally recognised are consequences of derangement of the relations between the different enzymes in the normal plant. Much has been written on the theme; it has already been considered in an earlier review (8). No change uniquely associated with infection has been found and the basis for distinguishing the different infections from one another by symptomatology probably depends on the distribution of the affected sites over the leaf and on the manner in which they spread more than on the actual extent of the change. Most of these changes are slow, but an increase in respiration rate and diminution in photosynthesis may be observed within an hour of infection (93). These changes are apparently not simply effects of the damage caused by inoculation for they do not follow inoculation with inactivated virus. A claim that inocula-

tion with nucleic acid prepared from apparently healthy pear leaves can change radically the composition of the leaf protein (65) is so surprising that it urgently deserves further testing.

It is clear that there has been, during the last few years, a welcome increase in interest in the detailed study of leaf enzymes and metabolism. As experimental material, leaves have the disadvantage that many of their enzyme activities are feeble compared to those of bacteria and animal tissues, that they are often difficult to disintegrate, and that they respond dramatically to changes in cultural conditions. The last quality makes it difficult to maintain a supply of uniform experimental material and causes much confusion in the literature. It does, however, give leaves great advantages when the coordination between different biochemical mechanisms is being studied; these advantages have not yet been adequately exploited.

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LIGHT-INDUCED REACTIONS OF BACTERIAL CHROMATOPHORES AND THEIR RELATION TO PHOTOSYNTHESIS[1,2,4]

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The photochemical reactions of intact photosynthetic bacteria have been treated in a number of excellent reviews by van Niel (1 to 4) and by Rabinowitch (5, 6, 7); a clear introduction to the subject has been given by Stanier and collaborators (8). To avoid duplication this review will restrict itself to a more limited topic within the field of bacterial photosynthesis. It will concern itself primarily with light-induced reactions carried out by cell-free, chlorophyll-containing particles from photosynthetic bacteria, termed chromatophores by Pardee, Schachman & Stanier (9). Wherever possible, comparisons will be made with information derived from the study of chloroplast reactions and tentative efforts will be made to relate photoreactions of cell-free preparations to photosynthetic activities of intact cells.

Knowledge in the field of cell-free bacterial photosynthesis at present is rather fragmentary, and preparations from only a few organisms have been investigated. Consequently, this review will have only a limited number of answers and it will raise many questions. It is hoped, nevertheless, that this paper will be able to point out that the study of bacterial chromatophore reactions may yield information of value for a better understanding of photosynthesis.

COMPOSITION AND STRUCTURE OF BACTERIAL CHROMATOPHORES

The chemical makeup of chlorophyll-containing extracts from photosynthetic bacteria attracted attention before information was available

[1] The survey of literature pertaining to this review was concluded in September 1958.

[2] The following abbreviations will be used: AMP (adenosine monophosphate); ADP (adenosine diphosphate); ATP (adenosine triphosphate); IMP (inosinic acid); IDP (inosine diphosphate); ITP (inosine triphosphate); DPN (oxidized diphosphopyridine nucleotide); DPNH (reduced diphosphopyridine nucleotide); TPN (oxidized triphosphopyridine nucleotide); TPNH (reduced triphosphopyridine nucleotide); FMN (oxidized flavin mononucleotide); $FMNH_2$ (reduced flavin mononucleotide); DPIP (2,6-dichlorophenol); PMS (phenazine methosulfate).

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[4] The organisms most commonly discussed in this review are: *Rhodospirillum rubrum* (*R. rubrum*), and species of *Rhodopseudomonas* belonging to the nonsulfur purple bacteria (family Athiorhodaceae); *Chromatium* sp., strain D (indicated simply as *Chromatium*) a member of the sulfur purple bacteria (family Thiorhodaceae); and *Chlorobium limicola*, a member of the green sulfur bacteria (family Chlorobacteriaceae).

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about the photochemical activity of such preparations. In 1925 Levy, Teissier & Wurmser (10) noted that the absorption characteristics of extracts obtained from *Chromatium okeinii* in the visible region of the spectrum corresponded to those of intact cells, supporting Lubimenko's suggestion that the photosynthetic pigments of photosynthetic bacteria most likely existed in the form of protein complexes (11). French (12) extended these observations to cell-free extracts from a number of nonsulfur purple bacteria. He found it impossible to fractionate extracts from a particular strain of organisms into chromoproteins of different color, and noted that the colored extracts had an isoelectric point at pH 3.7. French also described a light-sensitized oxidation of ascorbic acid which appears to be the first report of photochemical activity by a cell-free bacterial preparation (12, 13); this reaction will be discussed in more detail below. French also attempted to obtain CO_2 fixation with such extracts, but failed to do so (14). In 1939 Katz & Wassinck (15) reported their extensive observations on the spectral characteristics of algae and photosynthetic bacteria and of aqueous extracts obtained from these organisms; they concluded that the absorption curves of the aqueous extracts in the red and near infrared region closely resembled those of intact cells. They also obtained the same value as French for the isoelectric point of these chromoprotein complexes. Further spectral observations were made by French (16, 17) on extracts obtained from photosynthetic bacteria disrupted by supersonic vibrations; French concluded that this method of preparing cell-free extracts did not bring about appreciable changes in the absorption characteristics in the region from 450 to 900 mμ; he pointed out the usefulness of such preparations for the study of absorption spectra, since the cell-free preparations showed considerably less light scattering than intact cells.

In 1954 Pardee *et al.* (9) and Thomas (18) independently reported on electron microscope observations of extracts from *Rhodospirillum rubrum*. In each case the occurrence of particles about 1000 A in diameter was noted, and these particles were termed "chromatophores" by Pardee *et al.* and "grana" by Thomas. The present reviewer prefers the more noncommittal term chromatophore, because the term granum implies an analogy with chloroplast grana which has yet to be clearly established. Schachman *et al.* (19) have isolated these structures in relatively pure form by differential centrifugation (the particles have a sedimentation constant of 190 to 200 Svedberg units; one Svedberg unit is equal to 10^{-13} cm./sec./unit field). It was noted that all the photosynthetic pigments of the bacterial cells were present in this fraction. Such isolated chromatophores contained a small amount of pentosenucleic acid; deoxypentosenucleic acid was absent. Schachman *et al.* also made some calculations about the true size of these particles on the assumption that the chromatophores had been flattened out in the process of being spread on the electron microscope grid. Thus, they arrived at an actual diameter of 600 A for the chromatophore, a diameter for the anhydrous particle of 300 A, and an estimated mass for the hydrated

chromatophore of 30 million times that of the hydrogen atom. By making a count of the number of chromatophores released from a given number of cells, these investigators obtained a figure of 6000 chromatophores per cell which is in close agreement with their estimate of 5000 chromatophores per cell based on nitrogen analyses of cells and of chromatophores and on an estimate of the amount of nitrogen per chromatophore.

At present the most detailed chemical analyses have been carried out by Newton & Newton (20, 21) on the chromatophores of *Chromatium*. Bacteriochlorophyll occurred in these chromatophores at a concentration of 200 molecules per chromatophore (or one mole of bacteriochlorophyll per mole of protein of molecular weight 50,000). Newton & Newton detected large amounts of polysaccharides and an interesting ethanolamine phospholipoprotein; the latter protein contained glycerol, ethanolamine, and phosphorus in equimolar amounts with associated bacteriochlorophyll, carotenoids, and cytochromes in ratios of 10:5:1. These investigators also noted the presence of large amounts of acid soluble, nonheme, ferrous iron. The particles from *Chromatium*, furthermore, could be broken down into particles approximately one-half the original size which then contained twice the amounts of chlorophyll, carotenoids, cytochromes, and phospholipides per unit mass of protein compared with the original particles; acid soluble constituents (iron, phosphorus, pyridine nucleotides, and flavins) were present in smaller quantities. More recently, Newton & Newton (21, 22) have applied immunochemical techniques to the study of the chemical makeup of *Chromatium* chromatophores. They have produced antisera against chromatophores by the injection of chromatophores into rabbits and have found that these antisera contained antibodies which specifically reacted with isolated chromatophores. These antisera also inhibited photophosphorylation by *Chromatium* chromatophores.

A number of photosynthetic bacteria have been sectioned, and the sections have been examined under the electron microscope (23 to 26). Cells of *R. rubrum* grown in the light revealed remarkable differentation and structures could be observed, with diameters from 500 to 1000 A, comparable in abundance to the number of chromatophores estimated to be present in *R. rubrum* cells by Schachman *et al.* (19). Isolated and photochemically active chromatophores have been sectioned and examined and their appearance compared with the shape and size of the above structures in sectioned cells and found to be rather similar (25, 26). Vatter & Wolfe (24) also have sectioned light grown cells of *Rhodopseudomonas spheroides* which revealed vesicles 400 to 800 A in diameter, cells of *Chlorobium limicola* which showed vesicles 150 to 200 A in diameter, and cells of *Chromatium* which revealed vesicles 200 to 400 A in diameter. The latter values are in good agreement with a mean value of 320 A for the diameter of isolated *Chromatium* chromatophores (27, 28). Bergeron (28) has proposed a model for the structure of the *Chromatium* chromatophore consisting of a shell of lipide about 30 A in width surrounded by a protein shell about 60 A wide, with the photo-

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synthetic pigments distributed between the two layers. No assignment has been made in this model for components in the core of the chromatophore; sections of intact cells and of isolated chromatophores from *R. rubrum* have revealed however, that the chromatophores of this organism most likely are not hollow structures (26).

As indicated above, Newton & Newton (20, 21) have observed that isolated *Chromatium* chromatophores, when treated by sonic oscillation, yielded particles about one-half their original size but which contained twice as much bacteriochlorophyll on a protein basis. In the case of *R. rubrum* it has been possible to obtain small, pigmented particles, approximately 200 A in diameter by sonic treatment of cells in viscous media. These particles, on a chlorophyll basis, were fully as active photochemically as chromatophores isolated from comparable bacteria in less viscous media (25, 29). The 200 A particles could well be the structure which, according to Vatter & Wolfe (24), makes up the outer portion of the bacterial chromatophore. Young, light-grown cells of *R. rubrum* have failed to reveal chromatophores; instead, smaller structures with a diameter of 200 to 300 A have been observed scattered throughout the cells; fractionated extracts from such cells have revealed pigmented particles in this size range which were photochemically active (26). Extracts from dark-grown cells of *R. rubrum* did not contain chromatophores (19), and such extracts were found to be photochemically inactive or only weakly active (30). Thus, it appears certain that photochemical activity is associated with the presence of chromatophores or pigmented particles which, in the case of *R. rubrum*, are considerably smaller than the chromatophores, neither of which can be observed in dark-grown cells of this organism. Further investigations on the nature of the small, photochemically active particles should be of considerable interest.

PHOTOCATALYTIC ACTIVITIES OF BACTERIAL CHROMATOPHORES

Photo-oxidase

With the demonstration by Hill (31) of light-induced oxidation-reduction reactions catalyzed by chloroplasts, efforts were made to demonstrate similar reactions with cell-free preparations from photosynthetic bacteria. French, as mentioned earlier, was able to extract pigment-protein complexes from photosynthetic bacteria which would catalyze the oxidation of ascorbic acid by molecular oxygen when illuminated with visible or near infrared radiation. French studied some of the characteristics of this reaction, such as its dependence on light intensity, the effect of hydrogen-ion concentration, etc. (12, 13). Vernon & Kamen (32) extended these experiments and showed that extracts from *R. rubrum*, in addition to ascorbate, would photo-oxidize reduced mammalian cytochrome-*c*, or reduced DPIP. In the oxidation of ferrocytochrome-*c*, one mole of oxygen was consumed for each four moles of cytochrome-*c* oxidized. The reaction has a broad pH optimum from pH 6.5

to 7.5. The reaction was heat labile, although a good deal more stable to heat inactivation than a DPNH-cytochrome-*c* reductase present in the same preparations. Of the inhibitors tested, azide and hydroxylamine at concentrations of 10^{-2} *M* had no effect, cyanide showed a slight inhibition at 10^{-3} *M* and 82 per cent inhibition at 10^{-2} *M*, and mercuric ion caused 50 per cent inhibition at 10^{-4} *M*. In a later paper Vernon & Kamen (33) described a somewhat more complex system, containing DPIP and ascorbate, which resulted in a more rapid rate of photo-oxidation of ascorbate, two moles of ascorbate being oxidized for each mole of oxygen consumed. Upon the addition of excess catalase and ethanol to the reaction mixture, these investigators observed an undiminished initial rate of oxygen uptake, a simultaneous oxidation of ascorbate and of ethanol, and the termination of ethanol oxidation as soon as all the ascorbate has been oxidized. The authors concluded from the stoichiometry of the over-all reaction that the photo-oxidation of ascorbate and of ethanol were coupled, and they reasoned from indirect evidence that hydrogen peroxide could not have been formed as an intermediate of ascorbate photo-oxidation. They concluded that the reaction was not a simple photo-oxidation, but that it showed the manifestations of a bacterial Hill reaction in which water was split photochemically. Thus, the photochemical oxidant would react with ascorbate and the photochemical reductant with molecular oxygen, yielding an oxidizing agent which would bring about the oxidation of ethanol to acetaldehyde in the presence of excess catalase. This study was continued by Vernon & Ihnen (34) who observed that rates of photo-oxidation of ascorbate, in the presence of DPIP, could be stimulated further by the addition of FMN or by a number of other cofactors. White & Vernon (35) attempted to ascertain whether the light-induced inhibition of oxygen uptake by intact cells of *R. rubrum*, first observed by van Niel (1), could be explained on the basis of photo-oxidative inactivation of one or more of the respiratory enzymes. They observed that aerobic illumination of cell-free extracts would decrease the activity of the following enzymes present in the extracts: DPNH oxidase, DPNH-cytochrome-*c* reductase, and diaphorase; cytochrome oxidase activity was not affected. The authors did not indicate whether these *in vitro* light effects were reversible, as had been demonstrated for the *in vivo* light inhibition of O_2 uptake (36, 37). Ibanez & Lindstrom (38) have reported on the development of photo-oxidase activity upon illumination of dark-grown cells of *R. rubrum*. Extracts from dark-grown cells had only 5 per cent of the activity of light-grown cells; photo-oxidase activity increased upon illumination of intact cells with increased synthesis of chlorophyll and carotenoid pigments.

Most workers in the field probably agree that it is simple to obtain photo-oxidation activity with bacterial chromatophores, but not necessarily any other type of photochemical activity such as photophosphorylation. On the other hand, suppression of photo-oxidase activity is not simple and usually can be achieved only under highly anaerobic conditions. It would be most

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helpful if one could find means to inhibit photo-oxidase activity under aerobic conditions and still retain other photochemical activities, but at present it appears doubtful that this can be accomplished.

It has been questioned whether the photo-oxidase is truly an enzyme. Smith (39) has reported that the heating of *R. rubrum* extracts at 70° C. for 30 min. in 1.5 *M* sucrose did not completely destroy their ability to photo-oxidize reduced cytochrome-*c*. Chlorophyll alone can catalyze photo-oxidations (5, 6, 40), and chlorophyll associated with denatured proteins may well continue to catalyze such reactions as long as these complexes are maintained in solution and chlorophyll can retain intimate access to the reactants.

The suggestion has been made that the photo-oxidase may catalyze a "cheap type of photosynthesis" (33) resulting in the photo-oxidation of hydrogen donors with the production of high energy phosphate. This process, presumably, could occur at lower oxygen tensions than respiration and could be of metabolic importance in the absence of carbon dioxide. The inhibition of oxygen uptake by light (1) in the presence of carbon dioxide would appear to speak against a major metabolic role for such a process under normal conditions of photosynthesis.

Photophosphorylation

A light-induced esterification of orthophosphate with ADP to form ATP in the presence of cell-free preparations from photosynthetic bacteria, magnesium ions, and traces of suitable reducing agents was initially reported from the laboratory of F. Lipmann (41, 42, 46, 48). Such a reaction has been demonstrated with cell-free preparations from *Rhodospirillum rubrum* (29, 30, 38, 41 to 50), *Chromatium* (51 to 57), and *Chlorobium limicola* (51). The cell-free preparations from *R. rubrum* and from *Chromatium* have been fractionated and the highest activity found to be associated with the chromatophore fraction, or with the chlorophyll-containing small particle fraction from *R. rubrum* described earlier. Rates of photophosphorylation have been studied as a function of light intensity and a saturation curve has been obtained (50) comparable to light saturation curves for CO_2 fixation by intact organisms [(6), Chap. 28]. The pH optimum for photophosphorylation for both *Chromatium* (56) and *R. rubrum* (42) preparations is near pH 8 in a number of buffers. The photophosphorylation process is quite sensitive to heat inactivation; 2 min. at 80°C. will stop the process completely (46). Slow freezing and thawing of the extract also will lead to appreciable inactivation (46). Intact cells of *R. rubrum*, however, can be stored at −30°C. for several months and upon thawing, active chromatophores can be isolated from such stored cells. Isolated chromatophores of this organism can be stored in 40 per cent ethylene glycol at −15°C., retaining full activity for a number of months (42). Lyophilized *Chromatium* chromatophores, stored over P_2O_5 also retain their activity for several months (56).

Rates of photophosphorylation.—Rates as high as 350 μM orthophosphate esterified per hour per milligram of chlorophyll have been obtained with

chromatophores from *R. rubrum* in the presence of phenazine methosulfate (PMS) (45). These rates are about twice as high as the best rates obtained in the absence of PMS (29), but only about one-half as high as the best rates obtained with chloroplast preparations (58). For *Chromatium* chromatophores the best rates reported are from 50 to 100 μM orthophosphate esterified per hour per milligram chlorophyll (56).

Nucleotide specificity of the orthophosphate acceptor.—Washed chromatophores from *R. rubrum* or from *Chromatium* will actively esterify orthophosphate in the presence of ADP or IDP (48, 56), AMP is active only in the presence of traces of ATP or ADP and of added supernatant from the same bacterial extract which contains adenylate kinase (48, 56); IMP is completely inactive even in the presence of traces of either ATP or ITP and of such supernatant (48). ADP generally appears to be a better acceptor than IDP, and it may be of interest to establish whether the phosphorylation system can use either ADP or IDP directly as phosphate acceptors, or whether the system contains a transphosphorylase. Newton & Kamen (53) observed slight activity with AMP as phosphate acceptor in *Chromatium* preparations which could have been due to an incomplete removal of adenylate kinase from the chromatophores. These investigators also observed considerable incorporation of P^{32} from labeled orthophosphate into added ATP which is somewhat at variance with a later report by Anderson & Fuller (59) that little, if any, P^{32} exchange occurs with ATP from labeled orthophosphate during photophosphorylation by *Chromatium* chromatophores. The latter observation is in agreement with a report by Avron & Jagendorf (60) who noticed the absence of an exchange of P^{32} labeled orthophosphate with ATP by photochemically active chloroplasts. The absence of such an exchange is distinctly different from the behavior of animal mitochondria which carry out oxidative phosphorylation (61, 62). Newton & Kamen (53) also tested the incorporation of P^{32} labeled orthophosphate in the light into guanosine triphosphate and uridine triphosphate and observed only relatively small incorporations of labeled phosphate in these nucleotides.

Role of reducing agent.—There is no evidence for a conventional substrate requirement in photophosphorylation. Chromatophores can be washed to the point where little if any phosphorylation can be observed in the light unless traces of succinate, reduced DPN, or of other suitable electron donors are added (48). Under these conditions it can be demonstrated that as many as 100 or more molecules of orthophosphate are esterified for each molecule of succinate added (42). It also can be shown that the effectiveness of a given amount of reducing agent in eliciting photophosphorylation is a function of the concentration of molecular oxygen present in the system. The more anaerobic the system, the less reducing agent is required to bring about high initial rates of photophosphorylation (29, 49). Similar phenomena have been observed in the the process of photophosphorylation carried out by isolated chloroplasts (63, 64).

The function of added reducing agent in activating the photophosphory-

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lating process has been interpreted by Newton & Kamen (53) as the establishment of an "optimal steady-state relation between reduced and oxidized forms of interacting carriers." Any conditions, in turn, which favor photo-oxidation of the internal electron transport system may unpoise the system and bring about effective inhibition of photophosphorylation. Newton & Kamen (53) have observed that the most active redox reagents for the *Chromatium* system have potentials of approximately zero at pH 7, suggesting to them that *Chromatium* cytochrome with a potential (E_0') of -0.04 volt may be the component of the electron transport system which interacts with the external redox system. They also have pointed out that cytochromes of higher redox potentials are absent in *Chromatium* and have suggested that the *Chromatium* cytochrome may be the terminal component of the electron transport system which reacts with the photochemical oxidant. Similar, but not quite as specific suggestions have been made by other investigators (42, 48, 56).

Inhibitors of photophosphorylation.—Rather limited information is available on the effects of metabolic poisons and of redox reagents on the photophosphorylation process. Most of the observations below refer to the behavior of the photophosphorylation system of *R. rubrum* chromatophores.

Certain heavy metal poisons like CO (at 1 atm., tested in red light) (50), or 8-hydroxy quinoline at 10^{-3} *M* (42) have no effect; others like cyanide, azide, or *ortho*-phenanthroline give partial inhibitions at 10^{-3} *M* (42, 46). Mersalyl, a sulfhydryl reagent, is slightly inhibitory at 10^{-3} *M* (46) and arsenite, which can react with dithiol lipoic acid, is ineffective at 10^{-2} *M* (42). Fluoride at 10^{-2} *M* also has little or no effect (50).

Inhibitors of oxidative phosphorylation have been tested for their effects on photophosphorylation. 2,4-Dinitrophenol, at concentrations which will strongly inhibit oxidative phosphorylation (10^{-4} *M*) is relatively ineffective in photophosphorylation (42, 46); at higher concentrations (10^{-3} *M*) it becomes inhibitory (42). Dicumarol is a more effective inhibitor of photophosphorylation (50 per cent inhibition at 2×10^{-4} *M*); still more effective inhibitors are the butyl ester of 3, 5-diiodo-4-hydroxy benzoic acid (50 per cent inhibition at 3×10^{-5} *M*) (42), Antimycin-A (93 per cent inhibition at 4×10^{-7} *M*) (42), 3-hydroxyl, 1-heptyl quinoline-N-oxime (complete inhibition at 10^{-6} *M*) (44), and alkylated naphthoquinone SN 5949 (65) (68 per cent inhibition at 10^{-7} *M*) (42). Spectroscopic evidence indicates that Antimycin-A and 3-hydroxy, 1-heptyl quinoline-N-oxime act by blocking electron transport from cytochrome-*b* to cytochrome-c_2 (44), which is comparable to the action of Antimycin-A in oxidative phosphorylation (66).

A number of dyes like 2, 6-dichlorophenol indophenol, thionine, brilliant cresyl blue, and methylene blue are potent inhibitors of photophosphorylation under aerobic conditions, but are less effective under anaerobic conditions (42); their inhibitory effect under aerobic conditions most likely is due to their ability to accelerate photo-oxidation reactions (53). Pyocyanine and phenosafranine are reported to have little effect (42). The most interesting

dye is phenazine methosulfate (PMS) which inhibits photophosphorylation when the dye is kept entirely in its reduced state; however, when the dye is partially reduced it will in catalytic amounts accelerate the rate of photophosphorylation as first observed by Geller & Gregory (41, 42). Similar effects of PMS on the rate of photophosphorylation by *Chromatium* chromatophores have been observed by Newton & Kamen (53), and by workers in the field of chloroplast photophosphorylation (67).

Thus far no clear uncoupling effect on bacterial photophosphorylation has been demonstrated with any of the inhibitors listed. Such an uncoupling effect presumably would consist in the demonstration of an inhibition of photophosphorylation without inhibition (or with an acceleration) of light-induced electron transport. In chloroplasts, where electron transport can be followed more readily by the measurement of Hill reaction rates, such uncoupling effects have been demonstrated (68, 69).

Localization of photophosphorylation steps.—Thus far only one photophosphorylation site has been narrowed down in the bacterial system. Smith & Baltscheffsky (44) and Geller (42) have observed changes in the spectral characteristics of illuminated chromatophores of *R. rubrum* upon the addition of ADP in the presence of all required cofactors for photophosphorylation. The difference spectrum[5] obtained by Geller is difficult to interpret; the one obtained by Smith & Baltscheffsky, however, corresponds closely to the difference spectrum of oxidized minus reduced cytochrome-c_2 which can be obtained quite readily from the published absorption spectra of oxidized and reduced cytochrome-c_2 (70). The latter observation suggests that a phosphorylation site must exist between cytochrome-c_2 and the photochemical oxidant, and that light-induced oxidation of cytochrome-c_2 is coupled in an obligatory manner to a phosphorylation site. The effect of ADP on the oxidation level of cytochrome-c_2 in illuminated chromatophores is quite comparable to its effect on the oxidation level of cytochrome-c in rat liver mitochrondria under aerobic conditions, when mitochondria go from a nonphosphorylating state to a phosphorylating state upon the addition of ADP (71).

Evidence derived from the above spectroscopic observations, together with indirect evidence obtained from activation and inhibition phenomena, places an increased importance on cytochromes in bacterial photosynthesis, as emphasized for some time by Duysens (72), by Kamen (73), and by Chance & Smith (74).

Carotenoids and photophosphorylation.—Goodwin & Osman (75) have reported that diphenylamine will inhibit the synthesis of spirilloxanthin and of bacteriochlorophyll in *R. rubrum*, with the concomitant accumulation of small amounts of polyenes (37). Cohen-Bazire & Stanier (76) have repeated these experiments and have found a specific inhibition of carotenoid synthesis at low concentrations of diphenylamine (7×10^{-5} M). Under these

[5] [(light+ADP)−(light−ADP)]

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conditions carotenoid synthesis was completely inhibited, whereas chlorophyll synthesis and growth continued at about one half the initial rates; synthesis and accumulation of more saturated polyenes could be observed in these cells. Cultures of *R. rubrum* which were treated with diphenylamine and thus depleted of carotenoids showed a vastly increased sensitivity to photo-killing under aerobic conditions. This is consistent with the earlier findings of Griffith *et al.* (77), who ascribed an important physiological function to the carotenoids in protecting the photosynthetic apparatus from destruction by chlorophyll-sensitized photo-oxidation reactions. It is thus of considerable interest that Fuller & Anderson (55) were able to prepare carotenoid-free chromatophores from diphenylamine-treated *Chromatium* cells. Such chromatophores showed good photophosphorylation activity under anaerobic conditions, but lost this activity quite rapidly under aerobic conditions; photophosphorylation activity by normal, carotenoid-containing chromatophores was lost more slowly upon illumination under aerobic conditions.

Anderson & Fuller (57) also obtained action spectra for photophosphorylation by isolated *Chromatium* chromatophores. Qualitatively these action spectra corresponded to the absorption spectra of the chromatophores in the visible and near infrared region; the effectiveness of light absorbed by the carotenoids, however, was partially dependent on the method of preparation of the chromatophores. Best results were obtained when the chromatophores were prepared in 0.5 *M* sucrose, but even under these conditions utilization of light energy absorbed by the carotenoids appeared to be less than the 50 per cent value obtained from fluorescence measurements of intact cells (78). When chromatophores were prepared in more dilute solutions, light absorbed by the carotenoids was utilized even less efficiently in the process of photophosphorylation.

Thus carotenoids do not appear to be essential for the photophosphorylation process under anerobic conditions, and under aerobic conditions appear to have the same function ascribed to them for intact cells. However, light absorbed by the carotenoids apparently can be utilized in photophosphorylation but less efficiently than in photosynthesis.

Light-Induced Anaerobic Oxidation-reduction Reactions

In addition to photo-oxidation reactions which are catalyzed by illuminated bacterial chromatophores and which involve participation of molecular oxygen, a number of light-induced oxidation-reduction reactions have been studied under conditions where photo-oxidase activity usually has been greatly minimized.

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illuminated chromatophores showed an increase in absorption at 340 mμ which was ascribed to pyridine nucleotide reduction; additional spectral changes were ascribed to reduction of cytochrome components and of pseudohemoglobin [described by Vernon & Kamen (70)]. Smith & Baltscheffsky (44) at the same time reported the appearance of a peak at about 434 mμ in the light minus dark difference spectra of either aerobic or anaerobic extracts from *R. rubrum*; a similar peak could be obtained by the addition of hydrosulfite to the anaerobic extract. This peak, however, was not observed when the preparations were carrying out photophosphorylation. Duysens (79) also noted the appearance of an absorption band at 432 mμ upon illumination of intact cells of *R. rubrum* under aerobic conditions. He suggested that this change could have been due to an oxidation of bacteriochlorophyll. Chance and co-workers (80) obtained such an effect in the presence of phenyl mercuric acetate and this spectral change was interpreted either as the reduction of a hemoprotein or the oxidation of bacteriochlorophyll. The first possibility was considered less likely by these investigators because of the broadness of the absorption band and it was indicated that the interpretation of Duysens appeared to be more plausible. More recently, however, Olson & Kok have adduced evidence that this spectral change cannot be due to the oxidation of bacteriochlorophyll (80 b).

Specific cytochrome reactions.—More clear-cut information is available on the behavior of certain bacterial cytochromes upon illumination. In 1954 Duysens (72) described the anaerobic, light-induced oxidation of cytochrome-c_2 in intact cells of *R. rubrum*, a phenomenon which has been worked out in additional detail by Chance & Smith (74) and by Chance (81). Thus, it has been of considerable interest that a similar effect was observed by Smith & Baltscheffsky (45) in cell-free preparations from *R. rubrum* when, upon the addition of ADP, photophosphorylation was initiated in an illuminated system originally deficient in ADP. The observation of Duysens established the importance of cytochrome-c_2 for electron transport in photosynthesis by intact cells of *R. rubrum*, and the observations of Smith & Baltscheffsky demonstrated the connection between photophosphorylation and electron transport mediated by cytochrome-c_2. Smith & Baltscheffsky (45) also observed that in the presence of 3-hydroxy, 1-heptyl quinoline-N-oxime, or Antimycin-A, the light-induced oxidation of cytochrome-c_2 was accompanied by the reduction of a *b*-type cytochrome. Antimycin-A is known to block electron transport from cytochrome-*b* to cytochrome-*c* in animal mitochondria (66); thus the observation of Smith & Baltscheffsky can be interpreted as a demonstration of a bacterial Hill reaction, with oxidized and reduced components of the electron transport system accumulating, upon illumination, in spectroscopically recognizable quantities. Light-induced oxidation of cytochrome has also been demonstrated for intact cells of *Chromatium* by Olson (82) and by Olson & Chance (83). Olson & Chance (83) have estimated the quantum requirement for electron transport through the cytochrome system on the basis of the initial rates of change of absorp-

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in cell-free preparations of *R. rubrum* is localized completely in the bacterial chromatophores. It would be of great interest to learn whether this enzyme can mediate hydrogen transport in photoreactions of illuminated chromatophores.

Cohen-Bazire & Stanier (76) have postulated that chromatophores contain enzymes which can transform saturated polyenes to carotenoids; in carotenoid-depleted cells of *R. rubrum* this synthesis can occur either under aerobic conditions in the dark or anaerobically in the light. It should be worthwhile to ascertain whether carotenoids and bacteriochlorophyll could be formed in illuminated cell-free bacterial preparations, comparable to the synthesis of chlorophyll-*a* from protochlorophyll in cell-free systems from higher plants (108).

CONCLUSIONS

In examining the comparative biochemistry of chloroplast and chromatophore reactions one is surprised by the similarities in the photophosphorylation processes carried out by these diverse structures. Both in chloroplasts and in chromatophores photophosphorylation is essentially an anaerobic process which is coupled to electron transport between photochemical reductant and oxidant and which requires only catalytic amounts of external hydrogen donors. In addition, chromatophores, like chloroplasts, can carry out light-induced oxidation-reduction reactions which may result in the net accumulation of oxidized and reduced products. These observations lend strong support to van Niel's hypothesis that the primary photochemical reactions are basically the same in bacterial photosynthesis and in the photosynthesis of algae and higher plants. Admittedly, chloroplasts and chromatophores differ in their detailed chemical makeup and in the eventual fate of the photochemical oxidant in light-induced oxidation-reduction reactions; but it will remain for future investigations to elucidate the challenging problem of oxygen production by chloroplasts and the reasons for the absence of this process in bacterial systems.

The application of comparative biochemistry to problems of chromatophore and chloroplast reactions already has proved quite valuable. The specificity of ADP as phosphate acceptor and the stimulation of photophosphorylation by phenazine methosulfate (41) were effects first recognized for the bacterial system but found to hold equally true for the chloroplast system. The demonstration of photophosphorylation linked to electron transport via the cytochrome system in chromatophores may eventually find an analogy in chloroplast systems; on the other hand, phosphorylation linked to photoreduction of pyridine nucleotides described for chloroplasts (91) most likely should be searched for in chromatophore systems.

Further studies on the comparative biochemistry of chromatophore reactions and of photosynthesis carried out by intact bacteria also should prove to be of considerable value. It is of interest that already in 1948, Gest & Kamen (109) suggested the occurrence of a light-induced phosphorylation

based on studies of the photometabolism of intact cells of *Rhodospirillum rubrum*. Consequently, it should be worthwhile to amplify these studies and to elucidate the kinetics of high energy phosphate bond formation and utilization in bacterial photosynthesis. The light-induced oxidation of certain cytochromes is an additional phenomenon which links the photochemical activity of chromatophores to that of intact cells; nevertheless, it has not yet been demonstrated that phosphorylation in intact cells is coupled to the light-induced oxidation of cytochromes in the same manner as it occurs in chromatophores. The most striking similarity in the behavior of intact cells and of chromatophores is the requirement for stoichiometric amounts of reducing agent to bring about the photosynthetic reduction of CO_2 by intact bacteria and the photoreduction of DPN by isolated chromatophores.

Only a beginning has been made in the study of photochemical reactions of cell-free bacterial preparations. It is already apparent, however, that cell-free preparations carry out partial photoreactions of the intact organism, uncomplicated by respiratory processes of whole cells. Further detailed work on the comparative biochemistry of cell-free systems and of intact cells most likely will make worthwhile contributions toward a better understanding of bacterial photosynthesis as well as to our general knowledge of photosynthesis.

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THE STRUCTURE OF THE CHLOROPLAST

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Considerable research effort has recently been directed to elucidating the structure of the chloroplast—the site of photosynthesis in plant cells. In 1952 Weier & Stocking (1) reviewed the structure of the chloroplast together with its biochemical and genetic aspects as it had been studied during the previous ten years. They concluded that not only was there a lack of necessary experimental data, but also considerable confusion in the already existting data. Since 1952 improved techniques in microscopy, particularly in electron microscopy, chromatography, and other analytical methods have provided additional experimental information. This is evident from the numerous research papers and reviews on chloroplast structure and photosynthesis that have since appeared. Attention is therefore directed to the recent reviews by Frey-Wyssling (2), Granick (3), Mühlethaler (4), and Thomas (5, 6); to Rabinowitch for a historical as well as tabular summary of the data (7, 8, 9); and to a symposium of the Brookhaven National Laboratories, *The Photochemical Apparatus: Its Structure and Function* (10). In addition to these reviews, reference should be made to Leyon (11), von Wettstein (12) and Heitz (13) for discussions of more specific structural aspects of chloroplast development. Also to be noted are the reports of two recent symposia in the U.S.S.R., one on *The Origin of Life on Earth* (14), and the other the *Second All Union Conference on Photosynthesis* (15).

One hesitates, in this active field of research, to attempt to assess all of the accumulated experimental data or to review the recent excellent reviews. In gathering the information for this review three questions were paramount. (*a*) Can the structure of the chloroplast now be discussed at a molecular level? (*b*) What are the new data on the composition of the chloroplast and on the synthesis of its proteins and pigments? (*c*) How do these data relate to the growth and structural development of the chloroplast? It was with these considerations in mind that the recent available literature was searched.

The structure containing the photosynthetic pigments exists in a variety of shapes and sizes from the photosynthetic bacteria to the higher plants. These have been referred to as chromatophores, plastids, free grana, megaplasts and chloroplasts. This nomenclature was intended to give some idea of the plant photoreceptor structure as well as its phylogenetic position. Because of certain inconsistencies additional nomenclature has been introduced and suggested. In this discussion of the research literature it was found desirable to call all of these structures chloroplasts unless otherwise indicated or defined.

[1] The survey of literature pertaining to this review was concluded September 1, 1958.

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Methods

The techniques of microscopy—electron, polarization, fluorescence, phase, and interference—are the most direct approaches for the exploration of cellular structure. These methods are reviewed in *Physical Techniques in Biological Research* (16). The ability to see through the biological tissue section with the electron microscope rather than a shadow or replica of it is due mainly to progress in techniques by Drs. K. R. Porter and G. E. Palade of the Rockefeller Institute and to Dr. F. Sjöstrand of the Karolinska Institute (17, 18). This has greatly aided our research on the structure of the chloroplast. The method employs the use of a fixing agent which usually contains a metal which increases the electron density of the structure. After the biological tissue is fixed and embedded, sections are cut (.02 to .05 μ) sufficiently thin for electron penetration. The most successful fixative used is 1 per cent osmium tetroxide buffered with 0.028 *M* acetate-veronal at pH 7.0 to 8.0, although other fixatives such as potassium permanganate, potassium chromate and dichromate, uranyl nitrate and combinations of these have been reported. Almost all the recently published electron micrographs of the chloroplast are fixed with osmium tetroxide. The tendency to swell during fixation or postfixation treatment has been partially prevented by increasing the tonicity of the fixative with added sucrose (0.15 *M*). The embedding of the fixed material is usually carried out in an acrylic monomer (*n*-butyl methacrylate, methyl methacrylate or mixtures of these) which when polymerized possesses the right properties of hardness and ductility for sectioning with glass. Although the plant tissue may be well fixed, the necessity for dehydration with alcohols may lead to undersirable artifacts in the electron micrographs. In attempts to overcome these inadequacies, techniques in freeze-drying have been carefully investigated by Müller (19). Unfortunately the electron micrographs of the chloroplasts at present show no improvement; however, the method may be helpful in future investigation. Additional structural information has been gathered from light, polarization, fluorescence, and interference microscopy, where the chloroplast is viewed in its natural state. This information together with the structural detail obtained from fixed chloroplasts with the electron microscope gives us some idea of the microscopic "molecular" structure of the chloroplast.

Morphology

As already indicated, there is considerable variation in size and shape of the photosynthetic receptor. The recent general classification is based on the degree of internal organization as viewed with the electron microscope. In the photosynthetic bacteria and in particular, *Rhodospirillum rubrum*, the photosynthetic structures are referred to as chromatophores or as free grana. These structures contain bacteriochlorophyll as well as carotenoids and are small by comparison with the chloroplasts of the algae and higher plants. For example, the chloroplast of the algal flagellate *Euglena* is two to three times the size of the whole bacterium *R. rubrum*. In the blue-green

algae, too, no organized chloroplasts occur, but Niklowitz & Drews (20, 21) observed lamellae situated in the cytoplasm of the cells. In these structures neither the chloroplast membrane nor the stroma were observed and such structures are referred to as "chromatoplasm." It is, however, possible to isolate fractions from *R. rubrum* and a blue-green alga which show that the pigments are present in small particles 0.5 to 0.3 μ in diameter; these particles appear in the electron microscope as disc-like structures (22, 23, 24).

Two kinds of organized chloroplasts have been described: (*a*) the lamellar chloroplasts in the photosynthetic protozoa and most algae, and (*b*) the grana-containing chloroplasts of higher plants. The chloroplasts containing grana are also lamellar. These lamellae have been differentiated as the nonpigmented stroma lamellae and the pigmented chlorophyll-containing grana lamellae. In a few cases forms intermediate between both types of chloroplasts were observed, for example, in *Spirogyra* by Butterfasz (25) and in some Desmidiaceae by Chardard & Rouillier (26). [See discussion by Sager & Palade (27) since they take issue with the current terminology.] Found in most algae, but not in higher plants, is the pyrenoid. The pyrenoid is a differentiated part of the chloroplast generally associated with starch synthesis, but can also be a region of lipide storage. Leyon (11a) has suggested a functional and phylogenetic relationship between these two plastid components. The pyrenoids described in *Euglena* (29) and in *Spirogyra* and *Closterium* (11a) appear to contain lamellae continuous with those of the chloroplast.

There is now good evidence from electron microscopy of chloroplasts from a variety of plants that their gross morphology in various species may be different, but that in their ultra microstructure they consist of piled-up plates, more generally referred to as lamellae. However, additional experimental evidence as to the chemical nature of the fixed dense and less dense lamellae structures is needed. In certain organisms such as *Chlamydomonas*, whether grown in light or in darkness, Sager & Palade (27a) show a complex chloroplast organization of lamellae, pyrenoid, and eyespot within a limiting membrane. In the absence of chlorophyll, the lamellae are not formed, although the eyespot, starch grains, and certain rudimentary elements possibly related to the lamellae and the pyrenoid are still found within the intact chloroplast membrane. Similarly in *Euglena*, the lamellae are also dependent on chlorophyll (28, 29). Experimental evidence indicates that the photosynthetic pigments, the chlorophylls and carotenoids, are concentrated within the dense lamellae of the grana-containing chloroplasts and in the lamellar chloroplasts (30). Neither the individual pigment molecules nor the individual pigment macromolecules are, at present, within the resolving power of the electron microscope, and the exact orientation of the pigments within these layers is not known with certainty.

Frey-Wyssling proposed from his and Menke's previous studies on birefringence of the chloroplast that the chloroplast would consist of 20 to 30 parallel lipide layers separated from layers of aqueous protein by mono-

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molecular films of chlorophyll molecules; the hydrophillic porphyrin "head" of each chlorophyll molecule would extend into the aqueous protein complex and the lipophilic phytol "tail" would reach into the lipide layer (31). It was further postulated that each fatty layer would be 50 A thick, and that one layer would be separated from another by aqueous protein. The electron microscope has clearly established good qualitative proof of this picture. Studies with the algal flagellates *Euglena gracilis* and *Poteriochromonas stipitata* show double membraned plates or discs *ca.* 200 A thick whose dense peripheries are *ca.* 50 A thick with less dense interspaces of 200–500 A (28, 29, 32). Data have been collected for the average thickness of these dense chloroplast lamellae from a variety of species (11, 27). Electron micrograph measurements indicated the unit discs to be of the order of 100 to 300 A. In some chloroplasts low angle x-ray diffraction techniques, as demonstrated by Finean and co-workers in their studies of the fine structure of *Aspidistra* (33, 34), have corroborated the electron microscopic measurements showing a repeating unit of about 250 A. It was difficult to get good results with freshly isolated chloroplasts, but when the chloroplasts were fixed with osmic acid (OsO_4) good x-ray diffraction patterns were obtained. There is yet no chemical analyses of these layers. The location of the lipides, lipoproteins, and proteins has been assumed from the chemical reactions of the fixing agents and various stains with the biological material (30). The dense layers are referred to in the literature as either lipide or more preferably, lipoprotein, because of their affinity for the osmium tetroxide used in fixation. Frey-Wyssling & Steinman (35) pointed out that in good electron micrographs the lamellae can be seen to consist of spherical particles about 65 A in diameter and that these particles represent the protein macromolecules. These spherical particles may be a result of the fixation for electron microscopy, but it seems very likely that the lamellae are built up of macromolecules. There is experimental evidence to indicate that the chlorophyll molecules are preferentially oriented within the lamellae (36). From the geometry (diameter, length, and thickness of the dense and less dense layers) of the chloroplasts and the chlorophyll concentration per chloroplast, the cross-sectional area occupied by each chlorophyll molecule was calculated on the assumption that the chlorophyll molecules would be spread as monolayers. The space occupied by the chlorophyll molecules was found to be 222 A^2 for the *Euglena* chloroplast and 246 A^2 for the *Poteriochromonas* chloroplast (32). Similar calculations from microscopic data with similar results have since been made by Thomas and co-workers (37), Olson (10), and by Bergeron for the bacterium chromatophore (10). Goedheer similarly concluded from his studies of dichroism of anomalous dispersion of birefringence and polarization of fluorescence that chlorophyll resides in a monolayer at the lamellar surface (38). Although no definite assignment of the orientation of the chloroplast molecule was indicated within a lamina, there would be several possibilities as to the orientation of the chlorophyll molecule in the monolayer. He calculated that in *Meugotia* chloroplasts, the lamel-

lar area available per chlorophyll molecule is of the order of 250 A^2. Since the cross-sectional area of the porphyrin head of the chlorophyll molecule is known from x-ray studies to be about 225–242 A^2, these results would indicate that all the available chlorophyll molecules could be packed into the interfacial area.

It is of interest to compare the concentration of chlorophyll molecules within the chloroplasts for a variety of plant chloroplasts. Von Euler, Bergman & Hellstrom (7a) calculated that 1.7×10^9 molecules of chlorophyll are found in a single chloroplast of *Elodea densa*. This figure corresponds to a concentration of 0.1 mole per liter. Godnev & Kalishevich (7b) found that the leaf of *Mnium* had an average of 1.6×10^9 molecules of chlorophyll in each chloroplast. Since the volume of an average chloroplast of *Mnium* is 4.1×10^{-11} ml., the concentration of chlorophyll in it is 0.065 mole per liter. The corresponding figures for *Euglena* are 6.6×10^{-11} ml. and 0.025 mole per liter, and, for *Poteriochromonas*, 1.1×10^{-11} ml. and 0.016 moles per liter (32). Thomas, Minnaert & Elbers (37) have since studied and collected considerable additional data on chlorophyll concentration in the chloroplasts in a variety of species; from these data they conclude that although the morphology and structural dimensions of the chloroplasts vary widely, the mean area available in the monolayer per chlorophyll molecule is a constant of the order of 200 A^2, in agreement with the previously calculated values (32, 36, 38).

The average number of chlorophyll molecules in the interfacial monolayers was obtained by dividing the chlorophyll concentration by twice the number of lipoprotein layers. For *E. gracilis*, the number is 25×10^7 molecules per layer and for *Poteriochromonas stipitata*, 5.5×10^6. The number of chlorophyll molecules per unit area in the interfacial monolayers is just the reciprocal of the area available to each molecule, that is *ca.* 4×10^{13} per $cm.^2$ (32). If the porphyrin heads of the chlorophyll molecules were to lie at 0° like flat plates oriented as illustrated in Fig. 1a, they would be more efficient for trapping light than if they were stacked at 90° within the lamina, which would be the least efficient (orientation in Fig. 1b). It has been suggested by Trurnit, from chlorophyll monolayer studies, that the cholorphyll molecules would most likely lie at a 35–55° angle within the lamina, and Calvin has suggested, from calculations, that a 45° angle would be a closer approximation (10). It is very likely that the absorption oscillators of these pigment molecules are arranged with an orderly orientation in a way that maximum absorption will be observed for incident light polarized in a certain direction.

On the basis of the structural measurements from the electron micrographs, the pigment concentration, and calculations of the cross-sectional area available per chlorophyll molecule, a simplified schematic model was proposed by Wolken & Schwertz (32). The suggestion of Baas-Becking & Hanson (39) that four chlorophyll molecules are united to form tetrads, in which the reactive isocyclic rings turn toward each other, was employed. Interaction between the phytol tails was eliminated by arranging the

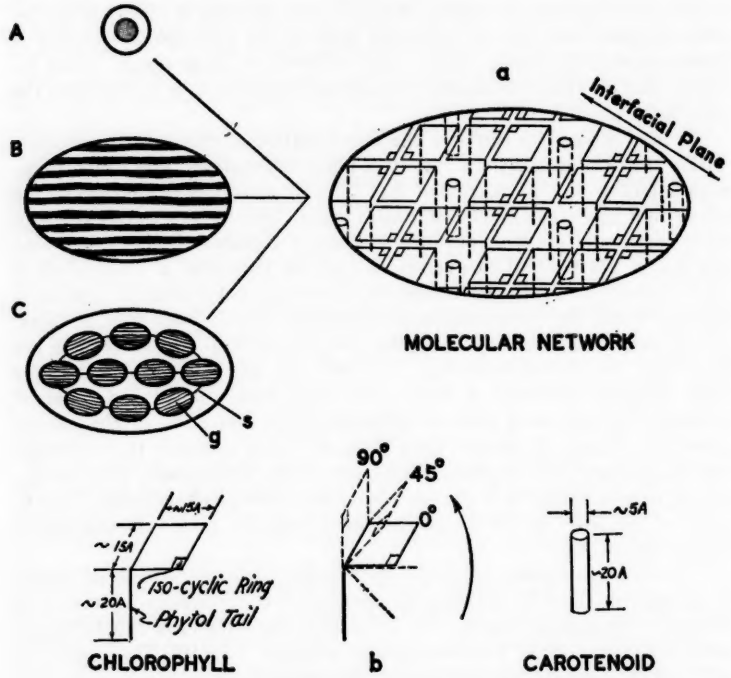

FIG. 1. SCHEMATIC STRUCTURAL REPRESENTATION OF CHLOROPLAST

The lamellae of B and the lamellae of the grana in C are the edges of the unit discs that make up these structures. (A, B, and C are not drawn to scale.)

A. Chromatophore, granalike chloroplast of photosynthetic bacteria and some blue-green algae. This is viewed by Bergeron (10) to have a dense core with the bacteriochlorophyll and carotenoid molecules oriented as a monolayer on the periphery.

B. Lamellar chloroplast of photosynthetic protozoa and algae. The dense bands in B represent the unit discs.

C. Chloroplast of higher plants containing grana (g), referred to as the pigmented lamellae, and (s), the nonpigmented stroma, or stroma lamellae. The lamellae of the grana and the stroma are continuous structures.

a. Representation of the molecular network in which a small area in the monolayer is greatly enlarged to show the packing of the chlorophyll and carotenoid molecules at the interfacial layers.

b. Indication of the possible orientation of the chlorophyll molecule as it may be packed in the pigment monolayer.

tetrads in such a way that one, and only one, of the phytol tails is located at each virtual intersection in the rectangular network. If the chlorophyll molecules were packed as monolayers as shown in Fig. 1a, there would be space available at the interstitial positions between them for the carotenoid pigment molecules. If these spaces are occupied as illustrated, there will be at least one carotenoid molecule for every three chlorophyll molecules in the network. The carotenoid molecules are slender linear molecules, probably less than 6 or 7 A in diameter, and therefore more than one molecule could conveniently fit in the holes formed by the chlorophyll tetrads, but this would lead to very tight fitting. Therefore a lower limit for the number of chlorophyll to carotenoid molecules of roughly one to one and a weight ratio of two to one is most likely. Since the molecular weights of the carotenoid molecules are one-half to two-thirds of the molecular weight of the chlorophyll molecule, a weight ratio, chlorophyll to carotenoid, of approximately 4:1 to 6:1 is to be expected, and is consistent with the available data given by Rabinowitch (7, 9). This close packing of the chlorophyll and carotenoid molecules in the pigment monolayer would permit energetic interaction between them. Similar chloroplast structural models with some variations have also been presented by Hodge, McLean & Mercer (40), Leyon (41), von Wettstein (12, 42), Steinman & Sjöstrand (43) and Bergeron for the bacterium chromatophore (10). Although the models at present can be used as a working hypothesis, additional analytical information is necessary.

How is the chloroplast's structure related to its functions as an energy transferring device? Is the photosynthetic efficiency greater for the more highly organized chloroplast and less so for the chromatophore of the photosynthetic bacteria? Such questions have yet to be resolved. As far as can be observed with the electron microscope any physical or chemical forces that affect the chemistry of chlorophyll synthesis or the chlorophyll molecule will disrupt the lamellar structure of the chloroplast and hence its photosynthetic activity (12, 28, 44, 45). The intact stroma lamellae also appear important for photosynthesis (46). An analogy to an emulsion has been indicated in which the lamellar structures are stabilized by the chlorophyll molecules (47). Arnold studied the photoconductivity of monolayers of chlorophyll and β-carotene spread on various surfaces. He found that chlorophyll plus β-carotene and chlorophyll or β-carotene alone are also photoconductive (10). It has been suggested from resonance studies that the photoreceptors would exhibit properties of crystals. Calvin and Arnold suggest that resonance studies and observations of the ordered structure of the chloroplast indicate that it is a kind of photo-battery, a semiconductor (48, 49, 50). This was also proposed independently by Terenin and his collaborators from other studies (15, 51). The chloroplast would exhibit many analogies to the Bell "sun battery." The assumption is that we are dealing with an electron-transfer phenomenon through many molecules by a conduc-

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tion band mechanism. It should be noted that a similar layered structure containing a carotenoid pigment-complex, rhodopsin, is found in the rod and cone outer segments of the visual cells of the retina and both function as light-trapping and energy-transferring devices.

Composition

Precise data on the chemical composition of chloroplasts are not available, since it is extremely difficult to obtain chloroplasts free from other cellular components (52). Techniques for the isolation of chloroplasts have been described by Clendenning (53). The chemical analysis of isolated chloroplasts from a variety of plants (including its enzymes and metals) is summarized by Rabinowitch (7, 9) and Frey-Wyssling (2, 31), and the enzymes of the plastids by Sissakian (64). For example, the chloroplasts of spinach (*Spinacia oleracea*), according to Menke (54), consist of 48 per cent protein and 35 per cent lipide. A range of values for protein from 41 to 55 per cent and for lipides from 18 to 37 per cent on a dry weight basis is given for a variety of plant chloroplasts. The chlorophylls and carotenoids represent about 5 to 10 per cent of the chloroplast, of which the chlorophyll concentration (as indicated in the earlier discussion of chloroplast morphology) is of the order of 5 to 6 per cent. With regard to the chemical nature of the various chloroplast pigments, reference should be made to the papers of Strain (55, 56) and, for the bacterial pigments, to Stanier (57). The chlorophylls and carotenoids are probably present within the dense lamina of the chloroplast not as free pigment molecules but complexed to a protein or a lipoprotein macromolecule. The question of the function of the carotenoids within the chloroplasts and its role in photosynthesis has been a debatable one. The carotenoids appear necessary for the function of the chloroplast according to Lynch & French (58). They found that when the chloroplasts were extracted with petroleum ether, β-carotene was removed and the chloroplasts were inactivated. On the addition of β-carotene the chloroplast activity was restored, thus indicating that β-carotene was an active component of the photochemical system. Fujimori & Livingston also postulated from studies of flash photolysis that carotene plays a direct part in the primary events of photosynthesis (59). However, Sager & Zalokar indicated from their studies of a pale green mutant strain of the alga *Chlamydomonas reinhardi* that the carotenoids are not essential for green plant photosynthesis, except perhaps in catalytic amounts and that carotenoids are more probably necessary for protection against photodynamic destruction (60). Two cytochromes, named cytochrome-*f* and cytochrome-b_3, have been found in the chloroplasts by Hill. One molecule of cytochrome-*f* was present per about 400 chlorophyll molecules (61). These two cytochromes together could make up as much as 20 per cent of the total protein content of the chloroplast. Nucleic acid is also assumed to be in the chloroplasts. The major part of the nucleic acid is RNA. The grana of higher plant chloroplasts yield stray ultraviolet absorption (62). Metzner (63) by en-

zymatic and staining techniques indicates that RNA and DNA exist in the chloroplasts. Sissakian (64a) has also shown that there is 0.3 to 3.5 per cent nucleic acids on a dry weight basis in the chloroplast. The mean total phosphorus is 0.39 per cent (as P) which must include the inorganic, phosphatide, and nucleic acid phosphorus. However, the methods of Metzner for the nucleic acid determination are considered inadequate and the values for DNA at present are in doubt (2). Synthesis of the peptide bond by chloroplasts has been studied by Sissakian using C^{14} and S^{35}. He found that this was linked to the enzyme activity of the chloroplast as well as to the mitochondria and concluded that peptide bond synthesis can be independently carried out by the chloroplast as well as the mitochondria. This research also claims to have confirmed that chloroplasts participate in protein synthesis. The nature and the properties of the plastid proteins do not remain constant in the course of growth, the kinds and concentrations of amino acids varying with the age of the plant. In reviewing the amino acid composition of the proteins of the chloroplasts of algae and higher plants they appear quite similar. There are 17 to 18 amino acids; these are aspartic and glutamic acids, serine, threonine, glycine, alanine, valine, leucine, isoleucine, phenylalanine, tyrosine, proline, tryptophan, methionine, cystine, arginine, histidine, and lysine. Quantitative data on the amino acids from the chloroplasts of various species is given by Sissakian (64, 64a).

Pigment Complex

The photosynthetic pigments, chlorophylls and carotenoids, in their natural state are bound to proteins or lipoproteins. Such complexes have been extracted from a variety of chloroplasts but not without difficulty, and are referred to here as chloroplastin. Moslova & Saponzhnikov (64) indicate that the stability of the chlorophyll-lipoprotein linkage changes during growth. A carotene-protein complex has also been isolated from the chloroplast of parsley and spinach (65). Osipova (64b) extracted the pigments and lipides from chloroplasts with polar and nonpolar solvents in order to determine the relationship between free chlorophyll (dissolved in the lipides) and the chlorophyll combined with the protein in the chloroplasts. Smith and his collaborators have isolated a physiologically active complex from the bean and a few other plants which they designate as a chlorophyll "holochrome," i.e., a pigment complex as it exists in its natural state (66, 67). The complex isolated was a protochlorophyll complex that is transformed to chlorophyll-*a* complex by light. From the chloroplasts of spinach, *Aspidistra*, *Nostoc*, and *Euglena*, a pigment complex, chloroplastin, was extracted with detergents (68 to 73). This method of extraction is similar to the extraction of the visual pigment complex, rhodopsin, from the retinal rods of the eye. The absorption spectrum of chloroplastin is nearer to the absorption spectrum of the living organisms, and appears homogeneous from its sedimentation in the analytical ultracentrifuge and its electrophoretic pattern (74). From the chloroplasts of *Euglena*, using 1 per cent recrystallized digitonin,

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a chloroplastin was extracted and its physical and chemical properties, such as kinetics of bleaching, rate of photoreduction of the dye, 2,6-dichlorobenzenoneindophenol, the evolution of oxygen, and the conversion of inorganic phosphate to organic phosphate were investigated (68, 75). In the visual pigment complex, rhodopsin, extracted from the retinal rods of the eye, a light-dependent reaction analogous to that occurring in the intact retina can be measured spectroscopically. This photochemical reaction "bleaches" rhodopsin and results in a shift of the absorption peak (from around 500 mμ to around 365 mμ). When chloroplastin was bleached by light no similar shift in spectra was observed. *Euglena* chloroplastin bleaches below 560 mμ by light energy alone; above 560 mμ it requires the combined effects of light and heat, with a steady decrease in optical density and the disappearance of the chlorophyll maximum absorption peak at 675 mμ. The total activation energy of bleaching was calculated to be 48.3 kcal./mole (68). In applying the Hill reaction with the dye, it was found that an absorption peak at 488 mμ (one of the *Euglena* carotenoid absorption peaks) increases in optical density in the light and then decreases in the dark to its original density. This reaction could be repeated several times by placing the reactants alternately in light and darkness without further addition of dye, indicating that there is an increase in the amount of carotenoid absorbing at 488 mμ simultaneously with the dye reduction. These studies indicated that the chloroplastin complex also had some physiological activity.

The molecular weight of spinach and *Aspidistra* chloroplastin, prepared with digitonin, has been calculated from its sedimentation constant as measured in the analytical ultracentrifuge to be of the order of 265,000 by Smith (70, 71, 72) and Smith & Pickels (76). This high estimate is due in part to the contribution of the detergent digitonin micelle. Miller & Anderson (77) and Smith & Pickels (76) have shown that digitonin forms micelles of minimal molecular weight equal to 75,000. Hubbard (78) has demonstrated that three such micellar units of digitonin are probably associated together with an average molecular weight of 225,000. If this molecular weight is subtracted from the average chloroplastin ultracentrifuge molecular weight of 265,000, a molecular weight of about 40,000 for chloroplastin would appear more probable. Takashima (79) crystallized a chlorophyll-lipoprotein complex with α-picoline from a leaf extract at low temperature; from diffusion studies its molecular weight was found to be 19,200 and it was further postulated that the complex would contain two molecules of chlorophyll per molecule of lipoprotein. Krasnovsky & Brin (80) consider the crystals isolated by Takashima to be contaminated with protein and other substances. The chlorophyll holochrome isolated by Smith and collaborators (81) from bean seedlings (*Phaseolus vulgaris*) in glycerine—KOH, pH 9.6) suggests a molecular weight of about 400,000. However, they did not determine the number of chlorophyll molecules or the chlorophyll to protein ratio in these complexes. The average molecular weight calculated from the geometry of the chloroplast and from the analytical data of the pigment concentration,

nitrogen, and dry weight of the complex from *Euglena* and *Poteriochromonas* is of the order of 21,000 to 40,000 (69, 74). This information, together with data obtained from the interference microscope on the wave path difference of the chloroplast in intact *Euglena*, was used to calculate a molecular weight of an average of 16,000 for *Euglena*. The analytical data also indicated that there would be one chlorophyll molecule to one macromolecule (74). The possibility that there may be two or more macromolecules cannot be excluded at present. Frey-Wyssling predicts that there would be 16 molecules of chlorophyll to one protein or lipoprotein macromolecule calculated on the basis that the macromolecule is 65 A in diameter; therefore the chlorophyll complex would have a molecular weight of 68,000 (2). All of these calculated values are for the most part in agreement as to the order of magnitude. However, differences in the molecular weight for various species could arise from the fact that the chlorophyll may be complexed with different proteins. How the chlorophyll molecule is complexed to the macromolecule is not as yet elucidated, nor do we know at present the chemical nature of the macromolecule itself.

Development

The chloroplast is considered to be a self-duplicating cytoplasmic organelle and is, therefore, of considerable interest in addition to its role in photosynthesis. To try to answer the question of how the chloroplast evolves from a proplastid or rudimentary structure to an organized functioning chloroplast is a most difficult task. There is little correlation of any morphological or phylogenetic changes with the biochemical properties of the chloroplast. Also, there is no agreement at present on the nature of the earliest stages in the proplastid development. It is noted that about 20 proplastids can produce as many as 120 chloroplasts by division. The chloroplast must be derived from some pre-existing chloroplast or proplastid. According to Strugger (82), the proplastid is a protoplasmic particle that always contains a primary granum, but Heitz & Maly (83) find that this granum is lacking in the youngest stages and appears only as a first step in the process of differentiation; finally, Mühlethaler (84) concludes from his electron micrographs, that the primary granum does not appear until later in development and is surrounded by protoplasmic material. But whether it occurs early or late in development the primary granum has been observed. This granum has a crystalline structure as demonstrated by Heitz and by Leyon in the electron microscope (13, 41, 84, 85, 86). Whether such crystalline macromolecules exist in the living state or whether they are enhanced by techniques for electron microscopy cannot be decided, but there is some speculation that the granum contains nucleoproteins. Therefore the evidence is suggestive that nucleoproteins are involved in the synthesis of the proteins of the chloroplast and its growth.

In *Euglena* which has become colorless after being grown in darkness, the chloroplasts disrupt and lose their lamellar structure. After long periods of

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time they are no longer recognizable. If such dark-adapted cells are brought back to light and their cytoplasmic structures studied at regular time intervals with the electron microscope, it is observed that after as little as 4 hr. of light exposure elongated bodies with the characteristic lamellae of the chloroplast are present. At the beginning, the lamellae are very thin, less numerous than later, and not tightly and regularly packed; with continued light the lamellae increase progressively in number and by 72 hr. of light exposure, the chloroplast has the shape and internal organization described for active *Euglena* chloroplasts (29). This study did not permit any definite conclusion concerning the origin of the apparently new chloroplasts, but chlorophyll synthesis was essential before the lamellar structure could be observed (28, 45). It was also indicated that a precursor porphyrinlike molecule may influence the synthesis of both carotenoids and chlorophylls (87).

Studies in the development of yellow plastids in the perianth of *Ranunculus repens* by electron microscopy showed that the structures characteristic of these yellow plastids are homogeneous globules up to 1500 A in diameter which first appear in young chloroplasts or leucoplasts (88). They are formed between the lamellae and during the process of development, as they increase in size and number, destroy the lamellar structure until at maturity only those droplets remain that line the inner surface of the plastid membrane. The development of the chloroplasts of flowering plants is discussed in detail by Leyon (11). The first stages to be seen in the development of these chloroplasts lack all the structural properties which are typical of the fully developed chloroplast. In development the distribution of the nonlaminar material in the chloroplast indicates a simple process of formation of new lamina and a relationship between the crystalline granum of the young chloroplasts to the formation of lamina. Heitz indicates that the crystalline structure of the primary granum, as found in *Chlorophytum*, *Aspidistra* and other monocotyledons, is also present in a dicotyledon (13, 83, 85). He also suggests that there must exist some connection between the crystalline lattice and the development of the lamellar stroma-grana structure as was suggested by Leyon (11, 41). Von Wettstein (12, 89) looked at the submicroscopic structure of the chloroplasts and employed chlorophyll lethals of barley. He found in the development of the barley chloroplast that: (*a*) there is an undifferentiated proplastid with a center core; (*b*) starch grains accumulate in the proplastids; (*c*) there is a formation of layers which protrude radially from the center; (*d*) the first pieces of lamellae are formed locally and at mutually independent points; (*e*) the short lamella multiply by a process of thickening and splitting; (*f*) these pieces of lamellae are connected in pairs that grow radially until they come into contact with one another; and (*g*) the pieces of lamellae fuse and the plastid as a whole becomes traversed by lamellae which then in turn multiply again. Essentially the process can be summarized as the formation of protrusions, folding, thickening and splitting, fusion, and then division. In an albino type essentially normal early proplastids enclosed by a double membrane were found. Differentiation did not proceed beyond

the earliest stages, but the plastid increased in size to that of a fullgrown chloroplast. The gene change apparently had blocked the development of the submicroscopic structure but not the growth of the plastid as a whole. In a yellow-green mutant referred to as xantha-3 the intermediate stages in development were blocked; instead of the formation of lamellae, globules accumulated in large masses, with a decrease in photosynthetic function. In the older stages of plastid development in this mutant the globules and inner structure of the plastid were broken down. The mutant showed no genetic block in pigment synthesis, although a greater concentration of chlorophyll-*b* was found in the globules; with destruction of the globules the pigment concentration also diminished. However, the picture of development from some precursor crystalline granum to an organized chloroplast still remains open for further experimentation.

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ACTIVE TRANSPORT OF SALT INTO PLANT TISSUE[1,2]

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The topic of active uptake and transport of salt in its many aspects has received meticulous and extensive treatment in recent years both in this journal (9, 27, 31, 72) and elsewhere (37, 83, 103, 104). Furthermore, a thoroughgoing review dealing specifically with accumulation and transport in plants has recently been published (93). What follows, therefore, is not meant to provide an addendum of the most recent contributions to the field, but rather represents an attempt to consolidate and interpret a variety of observations, some venerable, some new, dealing with certain specialized aspects of salt uptake and transport which are particularly plant physiological in nature. Consequently the treatment may often prove emphatic, if not all-inclusive, and will sometimes repeat in special context what others have said before. Absorption and uptake will be used as general terms describing ion and salt penetration of whatever kind into cells, tissues or organs. Active uptake and accumulation will imply metabolically implemented transport into the cell through a diffusion barrier, while adsorption will have the usual physical-chemical connotations which will be defined more explicitly in context.

The Prevalence and Meaning of the Initial Rapid Phase of Absorption

With the earliest extensive studies dealing with the time course of salt uptake into plant tissues, there has been recognition of the fact that a relatively brief interval of rapid uptake is normally followed by a slower but more prolonged period of absorption [(108,110); see Broyer (13)]. Although the full meaning of the separable absorptive stages, especially with regard to their interrelation, is still being elucidated, a spate of relatively recent work from several laboratories permits a more significant discussion of the matter than was possible but a short time ago. It can be said at once that whereas the significance of the prolonged, more or less steady-state uptake, classically denoted as phase 2, is generally agreed upon, a divergence of opinion attends the interpretation of the initial rapid uptake, known as phase 1. The outstanding question which currently merits attention is whether phase 2 is in any way dependent upon, or related to, phase 1.

In addition to the distinctions regarding the duration and velocity of the two phases, several features distinguish uptake as it occurs during the two

[1] The survey of literature pertaining to this review was concluded October 1, 1958.

[2] The following abbreviations will be used: AFS (apparent free space); DFS (Donnan free space); 2,4-D (2,4-dichlorophenoxyacetic acid); DNP (2,4-dinitrophenol); DPN (diphosphopyridine nucleotide); DPNH (diphosphopyridine nucleotide) (reduced form); WFS (water free space).

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periods. Thus, steady-state uptake is rigidly dependent on concomitant respiratory metabolism, succumbing to a variety of metabolic inhibitors—is in most instances an obligatorily aerobic process, has a high temperature coefficient, and is most often characterized by the absorption of both members of an ion pair. On the other hand the initial phase appears non-metabolic, may occur anaerobically, has a temperature coefficient typical of a physical process, and is predominantly concerned with cation absorption.

When uptake is described graphically as a function of time, an initial roughly hyperbolic stage is followed by a linear progression. It is proposed for convenience to call the first stage an absorption shoulder. The magnitude of the shoulder will be considered equal to the intercept of the line of regression of the linear part of the time course. The duration of the first stage, in turn, will be taken as the time at which the regression line diverges from the actual time-course curve. The duration of phase 1, as set out in Table I, must be considered a maximum estimate, since there are frequently no intervening measurements between t_0 and the time designated as the shoulder duration. By the same token the initial absorption rates constitute very rough estimates.

Much of the reason why the two-phased course of absorption has engendered so much interpretive difficulty is because there are at least six causes of nonlinearity. The first four may account for a rapid initial absorption, of the type most often described, while the last two provide some explanation for nonlinearity over a prolonged period.

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Salt saturation of the absorbing tissue.—The salt status of the absorbing tissue or organ has a profound effect on the salt absorbing capacity, as early indicated by Hoagland & Broyer (40). It has been effectively demonstrated that the salt status may change considerably during the experimental period as the result of absorption, and a change in the time course of absorption can occur for this reason [Sutcliffe (112)]. It is a matter of great interest, as will be seen, that the salt status exerts the observed effects on the absorptive process.

Table I brings together a variety of experiments designed for many purposes. The feature which they have in common is that the time course of absorption is characterized by two distinct phases. The sources from which the data of Table I are compiled indicate that absorption shoulders are clearly owing to different causes, and in many instances bear an intimate relation to the method whereby salt absorption is measured. For example, where absorption is measured by the change in conductivity of the external solution, phase 1 is measured in minutes, and the shoulder is in all probability due solely to diffusion into the WFS; whereas when absorption is measured by the loss from solution, or the gain by the tissue, of a specific ion, especially a cation, the shoulder is more prolonged and is in all probability due to adsorption exchange. Apart from the method of measurement, the factor which predominantly contributes to the shoulder depends in part upon the concentration; other things being equal, adsorption exchange plays a greater part at low external concentrations than at high ones (7, 26, 61).

Is Adsorption Exchange the First Step in Accumulation?

The thesis that ions penetrate impermeable cell membranes by combination with a carrier substance which can traverse these membranes has proven a provocative working hypothesis [see Epstein (27)]. Certain of the theoretical formulations based on this thesis have envisaged the initial step in salt uptake as the exchange of a cation from the external solution for a hydrogen ion arising from a cation carrier, and the exchange of an external anion for a hydroxyl ion arising from an anion carrier (49). The exchange of external cations for tissue cations, including H ions, has been shown to be rapid, extensive, and ubiquitous (10, 14, 50, 81, 87, 113). The significance of the exchange process has been obscured by the fact that on the one hand the observable exchange reaction has been considered the first step in ion accumulation [Jacobson *et al.* (49); Russell & Ayland (101); Gonzalez & Jenny (32); Higinbotham & Hanson (39); Lundegårdh (73)] while on the other it has been shown that the demonstrable exchange reaction is quite extraneous to the accumulation process [Epstein & Leggett (29)]. In distinction to overt ion exchange, however, a reversible combination of ion and carrier, deducible by kinetic analysis, has been shown to be intimately concerned with ion transport into the cell (27). There are several compelling rea-

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periods. Thus, steady-state uptake is rigidly dependent on concomitant respiratory metabolism, succumbing to a variety of metabolic inhibitors—is in most instances an obligatorily aerobic process, has a high temperature coefficient, and is most often characterized by the absorption of both members of an ion pair. On the other hand the initial phase appears non-metabolic, may occur anaerobically, has a temperature coefficient typical of a physical process, and is predominantly concerned with cation absorption.

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sons for believing that adsorption exchange as commonly measured (see Table I) has nothing to do with the accumulation process.

The corrected time course of accumulation.—Epstein & Leggett (29) in a study of the time course of absorption of Sr^{89} by excised barley roots, neatly distinguished between adsorption exchange and true accumulation by the simple expedient of removing all exchangeable Sr^{89} at the end of each absorption interval by exposing the tissue to nonradioactive Sr. When this was done, absorption proved a linear function of time from the beginning of the absorption period. Thus, while the adsorption-exchange reaction proceeds for at least half an hour, the accumulation rate is clearly independent of the saturation state of the adsorption exchange sites. Another implication which follows from these experiments is that diffusion penetration to the accumulation sites must be very rapid (cf. below). If the endogenous counter-ion is the same as that in the external solution, no shoulder is to be expected [cf. Stiles & Skelding (109)].

The relative specificities of adsorption exchange sites and carrier sites.—Whereas straightforward ion exchange is controlled primarily by the valence and position in the lyotropic series of the exchanging ions (1, 55, 56), the competition among ions for combination with ion carriers is characterized by considerable ion specificity [Epstein (25); Epstein & Hagen (28); Epstein Leggett (29, 65); Bange (6)]. In this light the inhibition of Ca uptake by K [Overstreet *et al.* (87)] and the noncompetitive interference with Sr absorption by K, Na and Mg (29), are to be considered as a process quite distinct from the competitive inhibition of K absorption by Rb (28), and the competitive interference with Sr uptake by Ca (29). To further emphasize the distinction, Middleton & Russell (81) have shown that in carrot disks Sr depresses Rb uptake in the initial stage of absorption (adsorption exchange), while exerting a markedly stimulating effect upon Rb uptake thereafter. Furthermore, the initial uptake of Sr alone exceeds that of Rb alone, while thereafter the rates of uptake are reversed.

Experiments by Sutcliffe (115) suggest that the considerable initial uptake of alkali metal cations by red beet disks involves nonspecific ion exchange, unrelated to accumulation. Thus, Rb, Cs, and Na all interfere with K uptake, while Rb, Cs, and K, interfere with Na absorption. All the alkali metals inhibit Li sorption. The relative rates of absorption of Na and K at 2°C. (adsorption exchange) are markedly different from the relative uptake of the two ions at 25°C. (exchange plus accumulation). In carrot disks the alkali metals all interfere with each other's initial absorption, and the alkaline earths in turn interfere among themselves (81). With beets again, Skelding & Rees (105) have shown that Ca seriously interferes with K uptake in the initial phase, but not during steady-state absorption. The former results are in marked contrast to those of Epstein & Hagen (28), and Epstein & Leggett (29), who, in experiments designed to elucidate carrier behavior, found not only that monovalent and divalent cations failed to interfere with each other, but also that competitiveness was quite specific

within each group. Bange (6) in work with maize seedlings involving Na and K uptake, affirms Epstein & Leggett's thesis. Further distinguishing between absorption in the initial phase and thereafter, Hanson & Bonner (36) have noted that Rb uptake by artichoke disks treated for 24 hr. with 2,4-D is twice that of the controls in the initial phase, and less than the controls during the steady state.

The ion affinities of carrier and of adsorption-exchange sites.—Since accumulation and adsorption can be readily distinguished by removing adsorbed cations at the end of each experimental period (29), the means are at hand to establish the apparent dissociation constant, K_s, for carrier-cation, and exchange site-cation respectively, and thereby further distinguish the two processes. Interestingly, Steward & Harrison (108), some twenty years ago, made the distinction alluded to above in a less direct way. After establishing that Rb uptake in phase 1 is essentially the same in live and in alcohol-killed potato disks [cf. Broyer & Overstreet (14)], they defined the relation of Rb uptake to external concentration in both types of tissue. The relation of absorption to concentration (hence the K_s) proved noticeably different in killed disks (adsorption exchange) and in live disks (adsorption exchange plus accumulation).

Concentration of sites.—The concentration of fixed negative charges on a fresh weight basis has been determined in a variety of plant tissues in several ways. The order of magnitude is evident from a limited number of observations: maize roots, 6×10^{-3} *M*, bean roots, 4×10^{-2} *M* [Hope (43)]; barley roots, oat roots, 2 to 6×10^{-2} *M* [Vervelde (121)]; cereal, tomato and flax roots, 10^{-3} to 10^{-2} *M* [Lundegårdh (72)]; potato disks, 4.8 to 6.0×10^{-2} *M* [Bange (5)]; red beet disks, 7 to 10×10^{-3} *M* [Sutcliffe (113, 114)]. In the past the estimated concentration has frequently been in error for reasons fully explained by Briggs (7). Furthermore, with respect to cation exchange measurements, it has been uncertain to what part of the total tissue volume the observed number of exchange sites should be ascribed. Briggs *et al.*, on the basis of an elegant analysis (7), have met the problem squarely (10). Beet disks were prepared so that exchange sites were entirely filled with a single species of cation. Unambiguous exchange measurements were then carried out which allowed the calculation of the total number of exchange sites per unit of tissue. In all instances, exchange followed the expectations of the Donnan distribution. This being the case, that fraction of the total volume constituting the Donnan space was ascertained, and the concentration of fixed charges within the DFS was thereupon calculated. The surprisingly high value of 0.6 *M* represents the fixed negative charge concentration (A) in the DFS, which in turn constitutes about 2 per cent of the total tissue volume.

Some interesting consequences arise from the high fixed-charge concentration in the DFS (7). Thus, when the concentration of an external uni-univalent salt is 5 per cent of A (0.03 *M* when A is 0.6 *M*), the quantity of anion entering the DFS is negligible; when the external concentration is

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10 per cent of A, the anion entering the DFS is but 1 per cent of A. It becomes evident that at external concentrations commonly used in salt absorption studies, anions, for all practical purposes, fail to penetrate the DFS. As a consequence it can justifiably be said that for anions, penetration of the DFS has nothing to do with accumulation—and from a logical point of view this must then apply to cations as well. Affirming this position are Epstein's experiments which show that anions are readily accumulated from the WFS, while cations virtually fail to enter the cell from the DFS (26, 29). Finally, since the ions of a uni-univalent salt are absorbed in essentially equal quantities during steady-state absorption (108) one may ask why, if adsorption exchange is a preliminary to accumulation, is there no evidence of appreciable anion exchange (61, 65)? The answer appears to be, once again, that adsorption exchange is unrelated to accumulation, and that the concentration of true accumulation sites must be low [see below, and cf. Lundegårdh (73, 75)].

Locus of adsorption-exchange sites.—The bulk of the fixed negative charges has been relegated to the cytoplasm by Lundegårdh (72, 75), Briggs *et al.* (10), and by others (17, 113). However, an alternative view of long standing suggests the cell wall may be the seat of the Donnan effect (54, 80, 124). The distinction is a most important one, since to attribute the adsorption exchange characteristics of plant cells to the cytoplasm is to imply that the outer membrane of the cytoplasm, the plasmalemma, is not a penetration barrier to ions. Two recent reports seem to establish unequivocally that the ion exchange characteristics of plant tissues are attributable to the unmethylated carboxyl groups of the cell wall pectin.

Keller & Deuel (54) have shown that the ion exchange capacity of ether-killed roots of wheat, maize, beans, tomato and tobacco ranges between 23 and 62 m. eq./100 gm. dry wt., and that 70 to 90 per cent of this capacity is due to the free pectin carboxyl groups. Jansen *et al.* (52) have compared the exchange capacity of cell wall preparations from *Avena* coleoptile sections with the exchange capacity of intact coleoptile sections as determined by Cooil & Bonner (21) and Tagawa & Bonner (116). The agreement is remarkable. The cation exchange capacity of fresh sections is 3.14×10^{-3} m. eq./gm. fr. wt., while the exchange capacity calculated from the determined exchange capacity of coleoptile wall material (0.18 m. eq./gm. dry wt.), and from the known prevalence of wall material (20 mg. wall/gm. fr. wt.), comes out to be 3.6×10^{-3} m. eq./gm. fr. wt. When the calculation is made on the basis of the known pectin free carboxyl concentration in the wall, the result is 3.3×10^{-3} m. eq./gm. fr. wt. In the light of these results there appears little reason to attribute the Donnan behavior of roots and disks of storage organs to the cytoplasm,[3] and the presumption remains reasonable that the plas-

[3] A. B. Hope and J. Dainty, in a manuscript submitted to the *Australian Journal of Biological Sciences*, report that the entire cation-exchange capacity of *Chara Australis* is located in the cell wall, and has a value of 0.3 m.eq./gm. cell wall, on a wet weight basis. The endogenous exchangeable counter-ion was shown to be Ca.

malemma represents a major permeation barrier to ion penetration [Walker (123)]. Briggs *et al.* (10) have estimated that the dissociable group which gives rise to the fixed charge in the Donnan phase of beet disks has a pK of about 3.0. This value is sufficiently close to the pK for pectic acid so that no contradiction is indicated. Bange (5) has demonstrated the amphoteric properties of potato disks (isoelectric point 2.25), which he associates with the surface of the protoplast, as distinct from the body of the cytoplasm. It seems possible that pectin carboxyl groups can provide the negative charges, while positive charges originate with another component [cf. Vervelde (122)].

Adsorption exchange and metabolism.—In vivid contrast to accumulation, adsorption exchange displays a temperature coefficient typical of a physical process (113), is independent of metabolic activity (13, 14), and may in fact take place in dead tissue (14, 108, 122). Withal, an interesting effect of KCN on adsorption exchange has been reported [Sutcliffe (113); Lundegårdh (73, 75)].

The ability of storage organ slices to accumulate salt, initially puny, increases sharply with time. The increase has been shown to be due to the development of a metabolism, which, in distinction to that of fresh slices, is capable of salt accumulation (62, 77). Adsorption exchange, in contrast, is essentially independent of the metabolic changes which occur with aging. Thus, Stiles & Dent (111) have noted a pronounced retardation of Mn absorption between phases 1 and 2 in carrot, mangold and swede slices. Since the retardation can be abolished by prolonging the preincubation period in water, it appears that the saddle in the time-course curve is due to the fact that the full adsorption-exchange capacity is present at once, while the full accumulation capacity develops only after some time. Although Hanson & Bonner (36) have shown that the initial uptake of Rb by slices of Jerusalem artichoke treated for 24 hr. with 2,4-D is twice that of untreated tissue, there is an augmentation of cell wall material in the presence of 2,4-D, and the additional cation exchange capacity is thus not surprising. Sutcliffe (113) has indicated a decrease in exchange capacity in beets with age, but he has assumed that absorption at 5°C. is perforce nonmetabolic, and in expressing exchangeability on a percentagewise basis he has related a more or less constant adsorption-exchange component to an ever-increasing part of the total absorption due to accumulation. It will be seen anon that bona fide accumulation can occur at temperatures close to 0°C.

Kinetics of absorption processes.—Table I suggests that when simple diffusive penetration into some part of the AFS is responsible for the absorption shoulder, the duration of phase 1 is most often measured in minutes. Contrarily, when absorption describes the transfer of a cation from the external solution to the tissue, the shoulder is commonly measured in hours. The diffusion coefficients of the common salts in water are such as to suggest rapid (minutes) penetration of solutes into the free space of thin disks and small-diameter roots. The rate of exchange reactions per se is taken to be almost instantaneous (55). Thus with the two rapid processes, diffusion and ex-

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change, in a catenary relationship, it would be expected that the overall process would be rapid.

Hope (43), Butler (17), and Briggs (7) have shown that penetration into roots and into disks is in accordance with Fick's laws [see Philip (88) for a general treatment of the subject]. Although one may anticipate that diffusion behavior should more nearly follow Fick's second law [Briggs (7)] than the first [Hope (43)], it is frequently not simple to make the distinction experimentally, since in the later stages of penetration the kinetics are very similar (7). Briggs *et al.* (10) discuss the movement of RbBr, RbI, and $CaCl_2$ into beet disks. The penetration of each ion follows diffusion expectations (Fick's second law). However, although the diffusion coefficients of RbCl and $CaCl_2$ at 5×10^{-3} M in water are respectively 1.995 and 1.179×10^{-5} cm.^{2}/sec. (97), and I and Cl behave similarly, the times taken to reach apparent diffusion equilibrium at 2°C., in millimeter-thick beet slices, are 90 min. for Rb, 200 min. for Ca, and 20 min. for Br or I. Since I is presented as the Rb salt, it is pertinent to enquire why Rb diffusion is ostensibly so much slower than that of I—and why absorption shoulders are so often of several hours duration when reasonable theoretical approximations suggest diffusion should be three-quarters complete in 75 sec. in a mm. slice (7).

Briggs (7) has made it clear that the AFS comprises a DFS and a WFS, the former being about 2 per cent of the fresh volume of beet disks, and the latter being about 20 per cent. Furthermore, the concentration of fixed negative charges in the DFS is as high as 0.6 M, and as a consequence, mobile anions are practically excluded from the DFS, and anion penetration is essentially into the WFS only. By the same token, the diffusion of a salt, as measured conductometrically, is primarily into the WFS under ordinary conditions, and the diffusion coefficient for anion and for salt penetration should resemble that for diffusion in water. Hope (43) and Briggs *et al.* (10) have found such to be essentially the case, the departures from exact identity being due mainly to the difference between the simple diffusion path assumed for the purposes of calculation, and the undeterminable devious diffusion path obtaining in the tissue.

Penetration into the DFS also follows diffusion kinetics. An additional feature must be considered, however, which is best understood by examining the diffusion of a radioactive cation into the DFS, where the counter-ion is the stable isotope. The attainment of equilibrium will, in this instance, depend not only on the rate of penetration, but also upon the establishment of equal specific activity, inside and out. For a case which follows Fick's first law, Briggs (7) offers the following formulation:

$$dS/dt = k\alpha(S_o - S_i)/\sigma.d.v.,$$

where $(S_o-S_i)/d$ is the concentration gradient of the radioisotope, S;—k is the diffusion coefficient , α the cross-section being traversed, v the volume of the DFS, and σ the ratio of the concentration of the ion in the DFS to that in the solution. It is evident that if σ is well above 1.0 the apparent dif-

fusion coefficient will be very much less than the actual diffusion coefficient. The cation concentration in the DFS has been shown to be as high as 0.6 *M*, from which it follows that the apparent diffusion coefficient will be 1/60 the actual in 0.01 *M* solution. If now, for the sheer joy of it, Rb is treated conceptually as an isotope of K, which will be considered as the endogenous counter-ion, there must be a redistribution of Rb and K until the "specific activities" are equal in the DFS and in the external solution. Diffusion kinetics will therefore follow the formulation for isotopic equilibration, and the time for attainment of apparent diffusion equilibrium will far exceed expectations based on the actual diffusion coefficient. Herein, it would appear, lies the explanation for the prolonged initial phases of absorption when cation exchange is involved.

In summary it is well to emphasize that the time course of the initial phase of penetration of anions and salts on the one hand, and cations on the other, describes movement into different areas, the one into the WFS and the other into the DFS. Cation penetration into the DFS of course proceeds through the WFS, and it is meaningless to speak of diffusion and adsorption exchange as alternative events in phase 1 (101). The kinetics observed will depend primarily on the slower event, and for cation penetration, for reasons discussed above, this will be equilibration of the DFS.

Leakage and Exchange Across the Permeability Barrier

It has become increasingly evident that an appreciable part of all plant cells is separated from the environment by a barrier through which the free diffusion of ions and highly polar molecules is prevented. Early studies (82, 91, 94) which ostensibly demonstrated an initial phase of diffusive permeation to an equality of concentration inside the tissue and out, were presumably in error because of the unrecognized effect of ion exchange on the conductivity of the external solution [Briggs (7); Robertson (92)]. Although the position is taken by some (9, 10, 17, 43, 113) that the permeation barrier is the tonoplast and that the cytoplasm is freely accessible to the environment, it is suggested herein that the cytoplasm is sequestered from its milieu by the plasmalemma,[4] a membrane with distinctly selective properties [Walker (123); Plowe (89); Hodge *et al.* (41); for a comprehensive treatment of membrane structure see J. D. Robertson (90)].

Leakage.—Whether the inner sanctum is considered to lie behind a surface membrane or behind the tonoplast, it is of interest to learn to what extent ions or salts can return to the environment, either by leakage or by exchange. Stenlid (106) has discussed leakage from plant tissues in its general aspects. Asprey (4), Stiles & Skelding (109), Steward (107), Robertson *et al.* (96), Davies & Wilkins (23) have all reported loss of ions from disks of storage organs under certain conditions. Early reports based on conductivity meas-

[4] See Buvat, R., and Lance, A., *Compt. rend.*, 245, 2083–85 (1957)—A paper which belatedly came to the author's attention—for outstanding electron micrographs of the plasmalemma.

urements are difficult to evaluate, since they fail to recognize the effect of adsorption exchange both upon the disappearance of given cations from solution, and upon the conductivity [Briggs (7)]. Nevertheless, there are cases where net loss from the tissue into distilled water or into dilute salt solutions has been determined by chemical or radioisotopic methods. Davies & Wilkins report the loss of 56 per cent (54 m. eq./Kg.) of the K of carrot disks, and 68 per cent (13 m. eq./Kg.) of the Cl, in five days of washing in distilled water. Loughman (67) notes the loss to water of 10 to 20 per cent of the total phosphate of fresh potato disks in several hours, the phosphate being entirely inorganic. In general, cation loss exceeds anion loss, and adsorption exchange of endogenous cations with external H must frequently be at issue in descriptions of leakage. For example, Steward (107) describes a considerable loss of Mg and K from potato disks during the first 25 hr. of washing, with no loss of Cl.

In any event, bona fide leakage appears to be slow,—as may be noted from a comparison of the rate of loss of Cl from carrot disks into water [circa 0.1 m. eq./Kg./hr. (96)] with the rate of uptake from 0.05 *M* KCL [3.3 m. eq./Kg./hr. (96)]. Whatever the rate of loss, it is of especial interest that leakiness is a characteristic of tissue slices freshly prepared from storage organs. When slices are washed in rather cold water, leakiness may persist for some time (107, 109, 111). At room temperature, however, leakiness soon ceases, the absence thereof being due to the change in metabolic pattern which attends incubation (62, 77). Beet disks bathed in running tap water at room temperature, accumulate large quantities of ions (76). Utilizing inhibitors of accumulation (DNP and KCN), Robertson *et al.* (96) have established that the trivial loss of ions from aged carrot disks is not the result of rapid reaccumulation, but is due rather to the absence of leakage. Hoagland & Broyer early noted that neither cyanide nor anaerobiosis evoke leakage in barley roots [Hoagland (40), cf., (14, 82, 94)]. Similarly, beet disks fail to lose salt in the presence of cyanide (95). It is interesting that chicory slices bathed in a continuously renewed solution of monovalent salt lose all aspects of semipermeability unless a trace of Ca is present (63). Comparably, barley roots lose considerable Rb to water in the absence of Ca, and very little in its presence (38).

Exchange across the permeation barrier.—Although the leakage of salts from tissue to the environment is neither common nor copious, exchange appears prevalent—and it is desirable to know whether exchange is limited to ions in the DFS (wherever one wishes to locate same), or includes regions behind the permeation barrier, in particular, the vacuole. In many instances, especially where the behavior of live and dead tissue is similar (14, 54, 108, 122), adsorption exchange is clearly responsible for the interchange noted. However, there are instances where exchange involves ions in the vacuole.

Briggs (8) has estimated the K interchange between the external solution and the vacuoles of carrot and beet disks from the data of Davies & Wilkins (23) and Sutcliffe (113) respectively. Carrot disks (10 gm./170 ml., 0.001 *M*

$K^{42}Br^{82}$) approach 60 per cent of isotopic equilibrium in five days. Beet disks (0.8 gm./4 ml. 0.02 M $K^{42}Br^{82}$) achieve 60 per cent equilibration with the external solution in one day. Both estimates are higher than those made by the authors. Briggs has formulated an expression for the estimation of the flux into and out of the diffusion-inaccessible space[5] of plant tissues as a function of time. Since for a given ion (K, in this case), the specific activity within the cell appears in the formulation, it is necessary to distinguish ions in the DFS from those in the cell.

After a relatively short time, the specific activity of K in the DFS can be assumed equal to that of the external solution. Since the DFS exchange capacity can be determined, as can the K in the WFS, an estimate can be made of the fraction of the total tissue K which belongs in the diffusion-inaccessible space, together with the specific activity thereof. From these particulars, together with the specific activity of the external solution, the flux into the cell ρ_i, can be derived from a series of graphical estimations which provide values for the expression:

$$d\sigma T/dt = R^*/K_T - \sigma_T R/K_T = (\sigma_o - \sigma_T)\rho_i/K_T,$$

where σ_o represents the specific activity of the external solution, σ_T that of the tissue; K_T the concentration of K in the tissue, and R and R^* the net flux of K and K^{42} respectively. Since $R^* = \sigma_o\rho_i - \sigma_T\rho_o$, ρ_o, the flux out of the cell, can be calculated. Briggs calculates ρ_i to be approximately constant from 6 to 18 hr., at about 4.2 m. eq./Kg. tissue, while ρ_o increases from 0.6 m. eq./Kg. at 6 hr., to 2.0 m. eq./Kg. at 18 hr. The external K concentration drops from 12 to 5.9 m. eq./l. in this time, while the tissue concentration rises from 74.8 to 108 m. eq./Kg. The per cent isotopic equilibration is 1 per cent in 6 hr., and 47.5 per cent in 18 hr.

Diffusion exchange [Ussing (118)], a process distinct from accumulation in being independent of metabolism, provides a theoretical means for the interchange of ions between the cell and the environment. However, there is no evidence for diffusion exchange in plant tissues. On the other hand, the diminution of net uptake with increasing internal concentration suggests that, however one-sided the equilibrium, the accumulation process is reversible. This contention is bolstered by the realization that it is not the over all salt status of the tissue which determines the rate of uptake, but rather the status with respect to the ion being absorbed [Humphries (46)]. In accord with the concept of reversibility, Briggs has found that influx and outflux are both affected by low temperatures and by KCN. However, Briggs reports that in saturated tissues where the net flux is diminished, the influx and outflux are both reduced. This observation is contrary to the position taken above, and a satisfactory answer is not at hand.

It is of interest that a variety of marine algae behave differently from the

[5] The diffusion-inaccessible space refers to the osmotic volume of Briggs (7, 9), and, in distinction to the DFS, will be spoken of as being within the cell. The specific activity of the tissue means the specific activity of the diffusion-inaccessible space.

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higher plants and much like many mammalian tissues [Krebs *et al.* (58); Briggs (7)], insofar as they exchange internal ions with the environment very rapidly. Scott & Haywood (102) using *Ulva*, describes a 75 per cent exchange of K with the environment in less than half an hour. Furthermore, the marine algae not only accumulate K, but also extrude Na, and both processes can be shown to be active. Studies with the red alga *Porphyra perforata* [Eppley (24)] are particularly interesting since this alga is nonvacuolate. In spite of this an osmotic volume of 45 per cent is indicated, which must represent cytoplasm behind a permeation barrier, the large remaining free space being primarily extracellular galactan sulfate with cation exchange properties.

MacRobbie & Dainty (78, 79) have performed a series of elegant studies with the marine algae *Rhodymenia palmata* and *Nitellopsis obtusa* which, if subject to generalization, will do much to reconcile the conflicting views of salt penetration into plant cells. Utilizing radioisotopes of selected ions, MacRobbie & Dainty estimated the influx and efflux separately of Na and K in *R. palmata*, and of Na, K, Rb, Cl, and Br in *N. obtusa*, with the algae kept in solutions simulating their natural environment. It proved possible to maintain the algae in solutions of radioisotopes for long periods, thereby introducing sufficient tracer to permit precise efflux measurements in subsequent periods. *N. obtusa*, having internodal cells up to 10 cm. in length and 0.8 mm. in diameter, proved suitable for the separate estimation of the flux in and out of the vacuole and cytoplasm respectively.

Kinetic analysis of the efflux from *N. obtusa* indicated three distinct stages;—an initial rapid stage associated with exchange with the AFS, a second stage with a half-time of less than 2 hr. representing exchange from the cytoplasm,—and a third stage, with a half-time of about 1000 hr., concerned with interchange with the vacuole. Thus, about 1.7 per cent of the vacuolar contents were exchanged in a day. The fluxes in *R. palmata* proved 100 times greater. The fluxes in and out of a cell in its natural environment were found to be equal.

In summary, the algal cells studied (and perhaps all plant cells) are presumed to have two membranes. The membrane between cytoplasm and environment resembles the membranes of animal cells, and is perhaps 20–100 times more permeable than the vacuolar membrane, which has the characteristics we have come to associate with the tonoplast. Cation discrimination has tentatively been relegated to the cytoplasmic membrane in *N. obtusa*, and involves Na extrusion, while the Cl accumulating mechanism has been assigned to the tonoplast. The picture as it emerges bears a striking resemblance to Arisz's conception of the permeation barriers in the cells of the leaves of *Vallisneria* (2, 3).

The Characterization and Estimation of True Carrier Sites

The concept of carrier-mediated transport of ions has arisen from the recognition that uptake is not a linear function of external concentration, but is best described by assuming a reversible association with some cellular

component (28, 84, 120). The most compelling evidence for ion carriers has arisen from kinetic analyses (27). In several instances, however, palpable observations have suggested not only that metabolically-created carriers exist, but that they may be used in a period subsequent to their formation; that is, in accordance with Asprey's early adumbration (4), transporting capacity may be stored.

Indirect intimations of carrier existence.—Russell *et al.* (98, 100) have come to the above conclusion on the basis of studies involving azide inhibition of phosphate uptake in barley roots, at decreasing concentrations of P^{32}-labelled phosphate. At very low concentrations of phosphate (0.001 p.p.m. P, 3.23×10^{-8} *M*) azide exerts no inhibitory effect on uptake into root and shoot, whereas at higher concentrations (10 p.p.m., 3.23×10^{-4} *M*) inhibition is appreciable. It was considered that at very low phosphate concentrations uptake is predominantly the consequence of the combination of phosphate with pre-existing carrier, this process being azide resistant. If the weight of roots (68) and the volume of solution in each experiment (99) is taken into account, it can be estimated that approximately 74 per cent of the environmental phosphate is absorbed in a 24-hr. period (from a 0.001 p.p.m. solution), and that the concentration of carrier in the roots is approximately 7×10^{-4} *M*, on the presumption that the entire uptake is due to combination with pre-existing carrier. The estimate is assuredly a maximal one. Although the foregoing interpretation is engaging, alternatives come to mind. For example, Chance & Hackett (33) have demonstrated that a considerable fraction of the terminal oxidase of a mitochondrial respiratory system may be combined with azide without causing a diminution in respiratory activity (when electron flux is low compared to total oxidase capacity). Also, there is the possibility of diffusive salt movement through the AFS directly into the xylem in that part of the root wherein the endodermis is as yet undeveloped [Crafts & Broyer (22)]. At low phosphate concentrations, where active uptake is minimized, the effect of free diffusion into the stele will be exaggerated.

Sutcliffe (113, 114) has postulated the participation of pre-existing carrier in uptake on the basis of the absorption of non-exchangeable K by beet disks at 5°C. However, there is plentiful evidence that metabolic absorption does not stop at 5°C. (13, 64, 75), and there is consequently little cause to impute uptake at 5°C. to pre-existing carrier. If the nonexchangeable K in Sutcliffe's experiments is taken as a measure of carrier concentration, the latter turns out to be almost 8×10^{-3} *M*, which, it appears, is a high estimate.

Kinetic estimation of carrier prevalence.—Recent papers by Lundegårdh dealing with the absorption of Cl and phosphate by potato disks (74, 75), and of Cl by wheat roots (73), purport to demonstrate anion adsorption sites, which are held responsible for the first phase of absorption. Absorption in phase 1, measured by the disappearance of anion from solution, proved temperature insensitive, and was complete in 15 min. The relation of initial uptake to concentration, estimated from the ordinate intercepts of Lunde-

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gårdh's Fig. 1 (73), was approximately linear in the range 2×10^{-3} M to 10^{-2} M. The calculated concentration of chloride adsorption sites in the cytoplasm, assuming the latter to be 5 per cent of the fresh weight, is as high as 0.18 M (9 μmoles Cl/gm. fr. wt.). Heretofore, anion binding sites, when demonstrable, were found to be in very low concentration (26, 48, 61, 86), in keeping with an earlier report of Lundegårdh's in which Cl absorption by wheat roots from a solution of 5×10^{-4} M KCl not only failed to exhibit a shoulder, but showed a lag (70). Except for an awkward numerical impasse, resting on one order of magnitude, the first stage of chloride penetration into wheat roots and potato disks resembles movement into the AFS, in accordance with Butler's observations (16, 17).

While Lundegårdh affirms the presence of a plasma membrane, he relegates both the cation and anion adsorption sites to the body of the cytoplasm, the anion sites being related to the cytochrome system of the mitochondria [(74), cf. Robertson (93)]. The ratio of negative to positive sites on the protoplast surface is considered to be about 100 (72), while the relative prevalence in the cytoplasm is considered equal (74). In the above view, penetration of ions into the cytoplasm is effected by "carrier rotation," is rapid, and presumably nonmetabolic. The rate-limiting step in accumulation is thought to be phase 1 adsorption (73, 75), a contention difficult to reconcile with the fact that phase 1 is temperature insensitive, while accumulation is decidedly temperature dependent. Lundegårdh has shown that KCN inhibits anion uptake in phase 1. The inhibition is ascribed to the effect of cyanide on the cytochrome system, which in Lundegårdh's view, is directly implicated in the acts of anion adsorption and accumulation (62). Since, nevertheless, data are presented [Fig. 2 (75)] which show that cyanide abolishes phase 1, with very little effect on phase 2, and since Sutcliffe (113) reports cyanide inhibition of K adsorption-exchange, the meaning of the cyanide effect in phase 1 remains moot.

Hagen & Hopkins (34) and Hagen, Leggett & Jackson (35) have essayed the characterization of the phosphate absorbing sites in barley roots with respect both to specificity and prevalence. The analysis is based on the presumption that the total uptake at any time is the sum of that which has been accumulated, plus that which is combined with the carrier site. Thus, $v=(MR)kt+(MR)$, where v represents the uptake in time t, k is the rate constant for accumulation, and MR represents the ion-carrier complex wherein M is the ion, and R the carrier. Uptake is seen to be a function of the concentration of ion-carrier complex, and it follows from the relation $[M]+[R]\rightleftharpoons[MR]$, and the assumption (28) that the breakdown of MR within the cell is a much slower process than the establishment of the depicted equilibrium, that $[MR]$ will equal $[R]$ at infinite phosphate concentration. The foregoing expression then becomes $V_{max}=[R]kt+[R]$.

As may be seen, the time course of absorption is described by a straight line, the intercept of which, at any external ion concentration, is $[MR]$. Since t may be set, v measured, and $[MR]$ obtained graphically, k may be

calculated. When k is once known, it may be inserted into the expression $V_{max}=[R]kt+[R]$, and since V_{max} may be estimated independently, $[R]$, the concentration of carrier site, may be determined. V_{max} is obtained from an elegant variant of the Lineweaver-Burk formulation of the Michaelis-Menten equation: $v=-K_M(v/M)+V_{max}$ [Hofstee (42)]. When v is plotted as a function of (v/M), the slope of the line yields K_M, the apparent dissociation constant of ion-carrier complex, and the intercept defines V_{max}.

In addition to the virtues above, the Hofstee plot indicates whether absorption is the consequence of a single type of ion-carrier interaction. Since the slope represents K_M, a departure from linearity indicates more than one ion-carrier combination. Hagen & Hopkins (34) have neatly shown that phosphate uptake occurs at two independent sites, and that one site is responsible for $H_2PO_4^-$ absorption while the other is concerned with $HPO_4^=$ uptake. The curvilinear graph obtained from a Hofstee plot when absorption is due to two ion-carrier interactions with disparate K_M's, may be resolved into two linear components, which by extrapolation yield V_{max} for the uptake of each phosphate species.

Hagen & Hopkins (34) first used relatively long absorption periods (3 hr.) in which the contribution of ion-carrier complex is small compared to the amount of phosphate accumulated. Hence estimations of $[R]$ and of k served mainly to establish the order of magnitude. Hagen *et al.* (35), however, followed absorption kinetics in five minute periods, wherein the contribution of the intercept component is relatively large. Under these conditions estimations of $[R]$ have much greater meaning. Happily for the quest, the dissociation constants for the two phosphate-carrier complexes proved vastly different, being 4×10^{-5} M and 5.5×10^{-10} for $H_2PO_4^-$ and $HPO_4^=$ respectively. Consequently, by selecting the proper phosphate concentration, it proved possible to study the absorption of $HPO_4^=$ alone, on the one hand, and of both species of phosphate ion, on the other. From these observations and a knowledge of V_{max} for each species, the individual rate constants and site concentrations were determined as described above. Thus R_a (for $H_2PO_4^-$) turned out to be 15.6×10^{-9} moles/gm. fr. wt. and k_a, 1.4×10^{-2} moles P/mole R_a/sec., while R_b (for $HPO_4^=$) proved to be 2.28×10^{-9} moles/gm. fr. wt, and k_b, 2.3×10^{-2} moles P/mole R_b/sec. It is evident that the concentration of carrier sites estimated in this way is several orders of magnitude lower than the concentration of purported anion-carrier sites reported by Lundegårdh, and the concentration of cation exchange sites described earlier.

It may be surmised that a considerable error would attend the above estimations if there were extraneous adsorption of phosphate. For then the formulation would be: $v=[MR]kt+[MR]+[adsorbed\ M]$. To avoid error roots were rinsed before each determination to wash out AFS phosphate, and it was established (private communication) that intercept and slope change proportionately with a change in concentration, a situation gainsaying a significant amount of extraneous adsorption. It is regrettable that the

designation v is used (instead of u for uptake, for example) where total uptake is meant, for inclusion of the intercept term deprives the symbol v of its classical connotation of a true velocity.

When the absorption of an ion follows Michaelis-Menten kinetics, it can be determined whether the absorption is competitively inhibited by the presence of another ion. In the Hofstee plot the slope (apparent K_M), will change, while the intercept (V_{max}) will remain the same in the presence of a competing ion. Fried & Noggle (30) have studied the absorption of K, Rb, Na, and Sr by barley roots, paying particular attention to the effect of H thereupon. The graph of v against (v/M) was distinctly curvilinear in each case, signifying two independent absorption sites, one facilitating absorption at low concentrations, while the other effecting uptake at considerably higher concentrations. The K_M's for the low concentration sites are approximately 3×10^{-6} M for K, Rb and Sr, and 1×10^{-5} M for Na, while for high concentration sites the K_M's range from 1 to 4×10^{-3} M. K and Rb were the only alkali cations tested for interaction, and they proved highly competitive. Furthermore, K_i, the dissociation constant of inhibitor-carrier, where K is considered an inhibitor of Rb absorption, proved to be the same as K_M for the absorption of K alone. The demonstration of competitiveness allows for the possibility that a cation is absorbed primarily at its own site, while competing to some extent at another site. However, when K_i equals K_M, absorption of both ions at but a single site is indicated, and the determination of K_i thus proves revealing [cf. Epstein & Hagen (28); Bange (6)]. Hydrogen ion was shown by Fried & Noggle to compete for the low concentration sites of the four cations studied, K_i for H being 1×10^{-5} M with regard to the absorption of K, Rb and Sr, and 2×10^{-6} M with regard to Na uptake. Unexpectedly, H competes with both alkali metal and alkaline earth cations, which groups presumably do not compete with each other [Epstein (27)]. A version of the algebraic equality adage might have been expected to apply here.

Sutcliffe (115) has taken issue with analyses based on Michaelis-Menten formulations, and contends that two ions absorbed at the same site need not interfere with each other, a contention which appears logically unacceptable. Sutcliffe offers the following expressions for absorption from a mixed solution of two cations, each at concentration X:

$$a_m = \tfrac{1}{2}(Pa/Pa + Pb)(a_2 + b_2)$$
$$b_m = \tfrac{1}{2}(Pb/Pa + Pb)(a_2 + b_2)$$

where a_m and b_m represent the uptake of the respective ions from a mixed solution, and a_2 and b_2 represent the uptake of each ion from a solution of a single cation at concentration $2X$. The term P describes the "preference" of a given site for each ion, which presumably bears some relation to K_M in the classical sense. If two ions are absorbed at the same site without mutual interference, the above expressions allow for two possibilities: either no distinction is made between the two ions, in which case the ratio of P terms is 1.0, or there is an exactly reciprocal interaction in which the less preferred

cation synergistically affects the uptake of the other—a possibility which though not excluded, must be satisfied by coincidence, since there is no reason to presume that synergism is obligatory. The fallacy of the formulation resides in arithmetically treating a_2 and b_2 as if they describe the absorption of one and the same ion instead of two. Although Sutcliffe offers one example where K appears to stimulate Na uptake, in almost all other instances the absorption of a given alkali cation is repressed by the presence of the others. The exception pertains to Li which fails to inhibit the uptake of Na, K, and Rb, but whose uptake is inhibited by the latter three, and by Cs as well.

Experimental demonstration of carrier synthesis and breakdown.—The case for ion carriers has so far been circumstantial, i.e. their presence has been deduced solely from kinetic considerations. Laties, although employing kinetic criteria for demonstrating the presence of chloride carrier,[6] has ostensibly been able to control the synthesis and destruction thereof in potato disks (64). The experiments hinge on the fact that the steady-state concentration of carrier is higher at room temperature than at 0°C. When disks with a history of 24 hr. at room temperature are plunged into a solution of KCl^{35} (0.04 *M*) at 0°C., an absorption shoulder of approximately 1 hr. duration is followed by an extended period in which absorption is relatively slow, and linear with time. The chloride taken up under these conditions, as measured by the radioactivity of rinsed disks, is neither washed out in water, nor exchanged away in nonradioactive KCl. If disks are kept in radioactive chloride at 0°C. until the initial hyperbolic absorption period is complete, and then transferred however briefly to water at 30°C., a new shoulder will be observed when disks are again placed into KCl^{35} at 0°C. Alternatively, if disks are kept for 1 hr. in water at 0°C. before exposure to chloride, no shoulder is perceptible. Dinitrophenol (DNP) prevents those events in the warm which lead to a new shoulder in the cold. The effect is reversible, and the potential for a new shoulder is developed at 30°C. as soon as disks are removed from DNP.

A tentative interpretation of the above observations is as follows. Since in the steady-state, carrier is regenerated as rapidly as it is broken down, an absorption shoulder due to carrier cannot be demonstrated at constant temperature. When tissue is transferred to a lower temperature, at which the steady-state carrier concentration is reduced, there is a short-lived surplus of carrier persisting from the original condition. The extra carrier, typical of the steady state at the higher temperature, is utilized in transport (the shoulder), following which a new steady state develops characteristic of the lower temperature. The saving grace is that the over all rate constant for carrier synthesis apparently exceeds that for destruction, allowing a measurable quantity of carrier (i.e., carrier precursor[6]) to be present in the steady-state. The quantity of true carrier must be very small, for both 30°C., and at

[6] The term carrier is here used operationally, indicating a unit of chloride absorptive capacity which can be created at one time and utilized at another. There is reason to believe that this latent absorptive capacity represents carrier precursor.

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0°C. where the shoulder has been previously removed, the graphical time course of accumulation regresses through the origin. The kinetics of carrier destruction in cold water mirror the time course of chloride uptake in the cold, and it appears that the rate-determining event involves the transport and decomposition of carrier, and not its loading. It is noteworthy that accumulation at 0°C. is real and prolonged just as in barley roots (13).

Carrier localization and characterization.—Hagen *et al.* (35) have made an attempt to define the nature and locus of phosphate carriers. Recognizing that salt accumulation is dependent upon the respiratory process of oxidative phosphorylation (62), they have studied the effect of inhibitors known to attack different loci in the electron transport chain, the seat of the initial phosphorylation reactions [Chance (19)]. The effect of each inhibitor was analyzed in terms of whether it was attributable primarily to a change in carrier concentration, change in rate constant, or both.

In brief, the site of $H_2PO_4^-$ absorption [R_a] was taken to be associated with DPNH, and the site of $HPO_4^=$ uptake [R_b] with reduced cytochrome *b*, on the basis that substances presumed to increase or decrease the steady-state concentration of DPNH and reduced cytochrome *b* respectively, were found to cause a corresponding increase or decrease in the absorption of the respective phosphate ions. A logical inconsistency raises a question about the interpretation of these experiments. First it must be noted that whenever analysis indicated that [R_a] or [R_b] was altered by the experimental treatment, both the intercept and the slope of the graphically depicted time course changed proportionately. This means that carrier function has been modified to the same extent as the concentration of carrier site. Carrier function directly depends upon electron flux [Hopkins (45)], i.e., upon the rate at which the designated member of the electron transport chain is alternately oxidized and reduced. It is therefore unexpected that amytal which lowers the flux, and azide, which at low concentration (10^{-5} *M*) either lowers or fails to affect electron transport, were found respectively to decrease and increase the absorption of $HPO_4^=$. Furthermore, it is illogical that amytal should inhibit $HPO_4^=$ uptake and fail to affect the absorption of $H_2PO_4^-$ when cytochrome *b* and DPN are sequential members of the same electron transport chain. Finally, although paraphenylenediamine, in reducing cytochrome *c*, causes the reduction of previous members of the electron transport chain including cytochrome *b*, it must reduce the electron flux through cytochrome *b*, and hence would be expected to decrease, and not increase $HPO_4^=$ absorption, as noted.

It is of no help in this dilemma to impute carrier function to intermediates linked to the electron transport chain, however many steps removed. Carrier function remains dependent upon electron flow. With regard for the vagaries which attend the interpretation of experiments involving the response of intact tissues to certain inhibitors and to substrates, and in view of the short absorption periods (five minutes) unaccompanied by concomitant respiratory or spectrophotometric measurements, the attempt to identify phos-

phate carriers with given stages of the respiratory electron chain appears commendable but overdrawn. With so much attention focused upon the role of cytochrome-mediated electron transfer in salt transport (62, 72) the mitochondria have naturally been suggested as possible transport vehicles. Although an ability to accumulate ions has been demonstrated in mitochondria [Robertson (93)] the consideration of mitochondria as carriers introduces more problems than it solves, and little that is revealing can be added at this time.

In connection with the two absorption sites reported by Hagen *et al.* it is of interest that Loughman (67), using a low phosphate concentration (10^{-5} *M*), found that freshly prepared potato disks fail to incorporate phosphate into nucleotides or into sugar phosphates, whereas Calo *et al.* (18), in an observation confirmed by Loughman, found that fresh potato disks exposed to 10^{-2} *M* phosphate readily incorporate inorganic phosphate into organic forms. After some hours, incorporation takes place readily at either concentration [cf. (69)]. The possibility exists that one phosphate absorption site functions in fresh tissue, whereas both sites function in aged disks. Although the ability to absorb phosphate increases markedly with age in potato slices, Loughman has determined that fresh slices absorb a small amount of phosphate from a 10^{-5} *M* solution, which remains as inorganic phosphate for some hours. In this case phosphate utilization, rather than absorption, may involve systems with markedly different phosphate affinities, although it is not ruled out that accumulation and certain aspects of utilization may be interdependent [cf. Hagen *et al.* (35); also Kylin (59)].

Does combination with carrier involve ion exchange?—Hagen *et al.* (35) and Fried & Noggle (30) have conceived the combination of carrier and ion in the most general way, and have purposely omitted an exchange reaction from their formulation [cf. Jacobson *et al.* (50)]. Although it has been suggested above that the exchange reactions which have been given the greatest attention are unrelated to the accumulation process, there is evidence that exchange accompanies bona fide accumulation under special conditions. Specifically, cations can be accumulated in excess of anions, and vice versa, depending upon the salt. For the maintenance of electrical neutrality the excess uptake of either ion must be compensated by a release of an ion of similar charge to the environment. The way in which the pH of the external solution changes with unequal uptake suggests that H ions, and OH or HCO_3 ions, exchange for external cations and anions respectively [Jacobson (47)]. Furthermore, unequal uptake is mirrored by an accommodating change in the internal organic acid concentration [Jacobson *et al.* (51)], excess cation absorption causing a rise, and excess anion uptake a decrease, in organic acids. A compelling question is why, if independent carriers implement cation and anion absorption respectively, does the uptake of a given ion depend upon the nature of its mate? Why, for example, should K uptake from a KCl solution be so much more rapid than from a solution of K_2SO_4? The answer may lie in the ability of the tissue to supply exchange ions metabolically when up-

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take is unequal. Since the limitation imposed by the exchange capability seems to apply only when there is unequal uptake, and not under conditions of rapid uptake when both ions of a salt are absorbed at roughly equal rates, it would appear that exchange is not an intrinsic aspect of the absorption process, but rather a secondary aspect related to the maintenance of electrical neutrality.

The organic acid (malate) formed in response to excess cation absorption is the result of dark CO_2 fixation (47, 51); an interesting ancillary question is why, in the presence of radioactive CO_2, does the malate which arises fail to equilibrate isotopically with the other organic acids of the tricarboxylic acid cycle [Jacobson (47)]. Either dark CO_2 fixation occurs extramitochondrially, away from the site of organic acid transformations within the tricarboxylic acid cycle, or malate is sequestered into the vacuole as rapidly as it is made by dark CO_2 fixation. The means whereby unequal uptake evokes organic acid changes remains to be elucidated.

Epilogue

The many facets of the absorption process discussed in the previous pages concern not only salt uptake into cells, but movement through tissues as well. The concept of cell compartmentalization which has emerged herein resembles the picture which Arisz offers on the basis of his studies of transport in *Vallisneria* leaves (2, 3). Specifically, Arisz suggests the plasmalemma and tonoplast are different in nature, and that salt penetration into the symplasm (cytoplasmic continuum) is an active process, albeit different from that which causes accumulation into the vacuole. With respect to the transport of salts into the stele, the consensus is that salts move in the symplast, and that the vacuoles represent a dead-end diversionary sump [Crafts & Broyer (22); Broyer (12); Lundegårdh (71)]. Cortical cells apparently serve a collecting function, funneling salts through the symplasm into the xylem—for absorption of ions through the endodermis is meager when the cortical cells are gently separated from the stele (71). The collecting function of the cortex depends upon the continuity of the cortical AFS with the environment, and is contrary to the notion that the root epidermis constitutes a diffusion barrier to the external solution [Epstein (26); Burström (15); Butler (17); cf. Lundegårdh (71)]. Estimates of the AFS are higher with excised roots than with roots of intact seedlings [(51), cf. (17)], suggesting that the AFS in intact roots does not extend into the stele. Although the AFS facilitates diffusion of salts into the cortex through short distances normal to the root surface, the evidence gainsays the AFS as a significant pathway for diffusive salt movement through considerable distances,—specifically, from root to shoot [Arisz (2, 3); Brouwer (11); Kylin & Hylmo (61); cf. Kramer (57)].

In the enduring theory of Crafts & Broyer [(22), cf. Lundegårdh (71)] salts leak from the symplast into the xylem and are not secreted therein [Russell (98)]. Recognizing that the concentration of salt in the xylem fluid

frequently exceeds that in the external solution, it follows that the cytoplasm is the initial seat of active uptake, and that it must perforce be separated from the environment by a diffusion barrier. By the same token, the cytoplasm cannot be part of the free space, else there would be no way to retain salts in the xylem against diffusion loss to the environment. It is noteworthy that with externally provided metabolite [2,4-D (53); phosphate (117)] it is the transient flux through the cytoplasm to which the tissue responds, and not the store of that metabolite in the vacuole.

In searching for an explanation for the selective leakiness [see Brouwer (11)] of cells within the stele, an analogy may be made between the postulated change in permeability of root cells between root surface and stele, and the changes observed in incubated slices of storage organs as one progresses from the surface to the center. In aged storage organ slices the surface cells vigorously accumulate salt, while the deep-seated layers continue to behave as fresh tissue, which not only fails to accumulate salt, but loses salt as well [Steward (107); Steward & Harrison (108)]. It has been shown that in potato slices it is neither the low O_2 nor the high CO_2 tension, but rather a volatile endogenous inhibitor which, in layers below the surface, prevents the development of the type of metabolism which can bring about salt accumulation (62). The situation may prove similar in roots. In sum, the movement of salts through tissues reflects transport behavior in cells.

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RESPIRATORY MECHANISMS IN HIGHER PLANTS[1,2,3]

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INTRODUCTION

Respiration, or "life with air," involves the breakdown and oxidation of organic compounds, the transfer of hydrogen (electrons) to molecular oxygen, and the release of utilizable energy within the cell. In this review, we will consider recent work on the biochemistry of these pathways and their regulation under various physiological conditions. The subject as a whole was last reviewed in this series in 1950 (118). Since then, specific aspects have been covered in many other reviews and two comprehensive treatments of plant respiration have appeared (164, 300). Only the work done since 1955 will be discussed here, and a few topics have been selected for special emphasis. During the period under review, evidence for the central role of the tricarboxylic acid cycle and the associated phosphorylations has been confirmed and extended. Interest in the electron-transferring chain has increased and some insight gained into the nature of cyanide- and carbon monoxide-resistant respiration. More attempts have been made to correlate respiratory mechanisms with physiological activities, and the structure of the respiratory apparatus has been studied in detail.

GENERAL ASPECTS, RESPIRATORY SUBSTRATES, AND INITIAL BREAKDOWN

Very few attempts have been made in the past to estimate the overall energetics of higher plant respiration. Using root tips and tissue cultures, Kandler (172) has obtained values of roughly one for the "synthetic efficiency," indicating that an equal number of moles of glucose were respired and assimilated. This value varied widely, depending on the age of the tissue and the presence of growth factors. The accepted role of oxygen as an electron acceptor in respiration has been demonstrated directly by showing the the respired CO_2 contains no label when O^{18}-oxygen is supplied to etiolated wheat sprouts, while it is labelled when O^{18}-water is supplied [Var-

[1] The survey of the literature pertaining to this review was concluded in July, 1958.

[2] The following abbreviations will be used: ADP, adenosine diphosphate; ATP, adenosine triphosphate; CoA, coenzyme A; DIECA, diethyldithiocarbamate; DNP, 2,4-dinitrophenol; DPN, diphosphopyridine nucleotide; DPNH, diphosphopyridine nucleotide (reduced form); EDTA, ethylenediamine tetraacetic acid; EMP, Embden-Meyerhof-Parnas; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; FP, flavoprotein; g, gravity; PEP, phosphoenolpyruvic acid; PN, pyridine nucleotide; R.Q., respiratory quotient; TCA, tricarboxylic acid; TPN, triphosphopyridine nucleotide; TPNH, triphosphopyridine nucleotide (reduced form).

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tapejyan & Kursanov (365)]. With respect to methods of determining the respiratory rate, a number of workers have pointed out the advantages of polarographic techniques (17, 188, 198, 270). The importance of eliminating diffusion limitations is illustrated by the recent demonstrations that an R.Q. close to one can be obtained with root tips [Betz (33)] and tobacco leaves [Landolt (190)] when the gas phase is oxygen; the higher values reported earlier were determined in air. Roots growing under conditions of suboptimal oxygen supply would be expected to have an R.Q. greater than one [Kandler (174)].

Carbohydrates and fats serve as the principal respiratory substrates. The metabolism of these components has recently been reviewed in detail (298, 299) and will be covered elsewhere in the present volume (113, 347). Major advances in this area have been made possible largely through the use of C^{14}-labelled intermediates, with the aid of which pathways of utilization and assimilation can be followed. For example, when C^{14}-glucose is fed to plant tissues, the respired CO_2 may come chiefly from either the added (342) or the endogenous (368) substrates, depending on the nature of the tissue; such a distinction would not have been possible in the older feeding experiments. Under certain conditions, as during cell breakdown, proteins may serve as respiratory substrates, but there is not in general a net utilization of proteins in plant respiration. Recent studies on the relationship between respiration and nitrogen metabolism, with special respect to the problem of protein turnover (341), were reviewed critically last year (382).

There is good evidence that plants may metabolize hexoses either via the EMP or the pentose cycle pathway, but the mechanism by which their respective contributions are regulated remains to be determined (16). The fact that the overall activity of the pentose cycle depends upon the presence of oxygen suggests that the rate of reoxidation of TPNH generated in the cycle may be the controlling factor. It is generally assumed that the EMP pathway is a "soluble system"; a similar distribution of the pentose cycle is suggested by the presence of the component enzymes in the $18{,}000 \times g$ supernatant fraction [Gibbs, Earl & Ritchie (115)], and by the stability of glucose-6-phosphate in the presence of mitochondria [Fritz & Naylor (110)]. Nevertheless, Servettaz (307) has demonstrated the pentose cycle enzymes in mitochondrial fractions prepared from pea stems and potato tubers, and further work on the localization is needed. The comparable enzymes of liver remain "soluble" after 2 hr. at $105{,}000 \times g$, but they are all sedimented together after 16 hr. at $144{,}000 \times g$ (266). The physiological importance of this cycle may well be related to its role in supplying pentoses and TPNH for cellular biosyntheses.

The Tricarboxylic Acid Cycle

Evidence continues to accumulate in support of the hypothesis that the main pathway of respiration in higher plants includes the TCA cycle; the earlier work was summarized by Krebs in 1954 (185). The initial breakdown

of carbohydrates and fats leads to the formation of pyruvate and acetyl-CoA, which are then completely oxidized in the cycle.

Tissue level studies.—When C^{14}-acetate is supplied to such tissues as wheat stems (242), barley roots (360), and cherry leaves (366), it is readily respired, and the distribution of C^{14} in the isolated organic and amino acids is consistent with the operation of the cycle as the principal oxidative pathway. With acetate-1-C^{14} and acetate-2-C^{14} it is possible to demonstrate the preferential conversion of C-1 to CO_2, as predicted by the cycle [Beevers (29); Doyle & Wang (82)]. The fate of labelled pyruvate in tomato fruits (52), barley roots (167), groundnut seedlings (102), and germinating castor beans (264) is also consistent with this scheme. It is frequently pointed out that the distribution of label in the various cycle acids is not equal. However, this fact alone does not argue against the operation of the cycle, since the kinetic inhomogeneity could be due either to secondary interactions of the acids with other systems or to their particular physical distribution within the cell. Thus, the unequal labelling found in succulent leaves fed with $C^{14}O_2$ is probably not due to the sluggishness of the cycle (54) nor to its inability to react rapidly after the isocitrate stage (258), but to the presence of a very active CO_2-fixing mechanism which is closely linked to the cycle. Saltman *et al.* (302) have recently followed the fate of $C^{14}O_2$ in *Bryophyllum* and they conclude that the TCA cycle plays a key role in succulent metabolism.

Malonate, the classical succinic dehydrogenase inhibitor, has been used frequently in the past to demonstrate participation *in vivo* of the TCA cycle (164). Reversible malonate inhibitions of potato slice (285) and barley root (167) respiration have been reported recently. It was reported earlier (89) that tobacco leaf respiration is 50 per cent inhibited by 0.01 M malonate, but Vickery & Palmer (367) have recently found that a much higher concentration (0.1 M) is required. At lower concentrations they observed a striking increase in the intracellular level of succinate, even though oxygen uptake was unaffected; this may account for the relative insensitivity to malonate. When some factor other than the TCA cycle activity is limiting, a partial inhibition of succinic dehydrogenase may not reduce the respiratory rate. This may be the case with the malonate-insensitive respiration of freshly-cut chicory root slices [Laties (192)] and wheat leaves [Farkas, Konrad & Király (96)], which become malonate-sensitive when the respiratory rate increases. The fact that malonate can be readily oxidized by plant systems may well affect its usefulness as an inhibitor [Giovanelli & Stumpf (116)].

Other agents have been used to block the TCA cycle *in vivo*. Fluoroacetate, which jams the cycle following the "lethal synthesis" of fluorocitrate, can block the respiration of plant tissues (132), including leaves (286). James (166) attributes the respiratory inhibition and citrate accumulation induced by 2,2′-dipyridyl to an effect on aconitase; however, in animal mitochondria this compound apparently acts on DPNH oxidation (381).

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Barker & Mapson (21) have shown that high concentrations of oxygen reduce the CO_2 output of potato tubers by blocking the cycle between citrate and α-ketoglutarate. Similar results have been obtained with pea seeds, where it is suggested that "oxygen poisoning" jams the cycle by inhibiting either aconitase or isocitric dehydrogenase [Turner & Quartley (361); Quartley & Turner (287)]. The effects of iron-deficiency and iron-toxicity on the levels of organic acids in leaves suggest that the TCA cycle is normally operative [DeKock & Morrison (79)].

Cell fraction studies.—The initial work which established plant mitochondria as the organized centers of respiratory activity was reviewed in detail in 1955 (126). Since then, the list of plant tissues from which particles exhibiting TCA cycle activity have been obtained has grown so greatly as to support the view that the system is ubiquitous in higher plants. Laties (192) has recently listed many of these tissues and others will be mentioned throughout this review. Particles have been obtained from representatives of the major classes of vascular plants (Filicineae, Gymnospermae, Angiospermae), from seedling tissues, roots, stems, leaves, flowering parts, fruits, and storage tissues. In view of previous difficulties (51), it is of special interest that leaves will yield active preparations. From both etiolated and green pea leaves, Smillie (326, 327) was able to isolate particles which oxidized the cycle acids and converted fumarate to succinate in the presence of malonate, thus showing that the complete cycle was operative. His subsequent analyses of the chemical and enzymic properties of fractions sedimented from leaf homogenates demonstrated that this activity is associated with particles which correspond in all respects to the mitochondria of other tissues (328, 329, 330). This conclusion is supported by the recent separation and characterization of chloroplasts and mitochondria from broad bean and Swiss chard leaves; only the mitochondria showed TCA cycle activity [James & Das (168)]. The mitochondria, which are present throughout leaves grown either in the dark or in the light, presumably serve as the major respiratory centers. Ohmura (272) attributed the oxidation of TCA cycle acids by a spinach leaf particulate fraction to the chloroplast fragments, but it is most likely that the active particles were mitochondria. Other green (or partially etiolated) leaves which have been used to prepare mitochondria include those of ferns, cabbage, and lettuce [Freebairn & Remmert (104)]; a detailed study of the cabbage particulate preparation showed the operation of the complete TCA cycle (105). All of this evidence, combined with that obtained at the tissue level in tracer and inhibitor experiments, strongly suggests that the TCA cycle is normally operative in green leaves, as it is in other plant parts.

The method used for isolating mitochondria is critical in all these studies, and some recent modifications of the original techniques have proven helpful. By carefully controlling the speed and time of blending, it has been possible to prepare active particles from Waring blender homogenates (28, 30, 37). The inclusion of EDTA, an effective chelating agent, in the isolation medium can markedly promote the oxidative activity [Tager (353)], and

this practice has been widely adopted. The EDTA must be present during the isolation, but it can subsequently be removed without deleterious effects [Lieberman & Biale (200)]. With acid tissues, such as the apple fruit, an alkaline isolation medium (pH 9) may be necessary for the demonstration of optimum particle activity [Lieberman (199)]. Specific stimulatory effects of phosphate in the isolation medium have been reported in some, but not all, cases (28, 37, 86, 350). A reducing agent, such as cysteine, has frequently been added to prevent oxidative reactions; in potato homogenates, this inhibits a soluble DPNH oxidizing system, probably by removing the quinones [Hackett (131)]. A method for carrying out the isolation under anaerobic conditions has been described [Cohen *et al.* (65)]. The deleterious effect of lecithinase may be prevented by fluoride [Goodwin & Waygood (121)]. Chloroplasts and mitochondria have been effectively separated by centrifugation in a density gradient [Jagendorf (162); James & Das (168)]. The preliminary vacuum-infiltration of intact roots with a sucrose-EDTA solution can greatly enhance the succinoxidase activity of the subsequently extracted particles [Ducet, Vandewalle, & Shealtiel (87)].

Some recent studies have dealt with mechanisms of the TCA cycle reactions in isolated mitochondria. The fate of C^{14} has been followed when pyruvate-2-C^{14}, acetate-2-C^{14} and succinate-2-C^{14} are oxidized (105, 336, 337). A comparison of the $C^{14}O_2$ data with measurements of O_2 and CO_2 exchange during succinate oxidation showed that endogenous substrates had also been oxidized, thus emphasizing that O_2 uptake results alone do not give a definitive indication of the amount of added substrate respired [Freebairn & Remmert (105)]. The operation of the cycle has been demonstrated by chromatographic characterizations of the reaction products [Avron & Biale (15); Lieberman & Biale (202)]. Pyruvate and α-ketoglutarate oxidation are regularly promoted by additions of adenylate, magnesium, and cocarboxylase, while the effects with DPN and CoA have been varied. The whole spectrum of cofactor requirements, including the need for an acetyl-CoA acceptor during pyruvate oxidation, has been shown clearly with castor bean and potato mitochondria [Beevers & Walker (30); Walker & Beevers (370)]. It now seems most likely that all the cycle oxidations (except succinate) are linked to DPN, since a DPN-specific isocitric dehydrogenase has been demonstrated in plant mitochondria [Davies (75)]; the enzyme contains essential —SH groups and shows a manganese requirement, which may account for the specific manganese stimulation of citrate oxidation (28). There is also a TPN-isocitric dehydrogenase, which is localized primarily in the soluble fraction, but this may be more closely related to reductive reactions. It is frequently difficult to demonstrate the oxidation of oxalacetate by plant mitochondria, and this is probably due to its ability to compete successfully with the respiratory chain for hydrogen [Walker & Beevers (370); Avron & Biale (14)]. Oxalacetate can also inhibit succinate oxidation. Substrate oxidations may be difficult to demonstrate when the particles have a high endogenous respiration (104, 281).

Related organic and amino acid metabolism.—The metabolism of organic

acids in plants involves a variety of reactions which are closely linked to the TCA cycle. For example, the glyoxylate cycle reactions may replace or bypass the steps from isocitric to malic in the TCA cycle [Kornberg & Beevers (184)]. The fact that isocitritase and malate synthetase are present in the $25{,}000 \times g$ supernatant suggests that these reactions may be physically separated from the mitochondrial system. A reversal of the isocitritase reaction might contribute to the accumulation of citrate and isocitrate. Acid accumulation in plants is related to the various dark CO_2-fixation reactions, of which that catalyzed by PEP carboxylase is probably the most important (301, 371). The PEP itself may be generated in the mitochondria as a result of the oxidation of TCA cycle acids [Bandurski & Lipmann (20)]. Both PEP carboxylase and PEP carboxykinase (oxalacetic carboxylase) are present in the mitochondrial and soluble fractions [Davies (76); Mazelis & Vennesland (241)]. The close relationship between dark CO_2-fixations and respiration is emphasized by the dependence of fixation on the partial pressure of oxygen [Moyse (257)].

Plant mitochondria are able to oxidize amino acids, and particularly glutamate, via the TCA cycle (101, 105, 288, 326). Both transaminase and glutamic dehydrogenase are present on these particles. When labelled glutamate is fed to plant tissues, it is readily degraded to respiratory CO_2 (263, 324, 342). Direct evidence that the carbon skeletons of amino acids are derived from TCA cycle intermediates has been obtained both with mitochondria (105) and intact tissues (203).

The mitochondria can oxidize a wide variety of other compounds, including sulfite (356), glutathione (387), lecithin (104), L-galactono-γ-lactone (218), α-glycerophosphate (346), tartaric (335), phenylpyruvic (68), and malonic acids (116, 388). The oxidation of fatty acids is considered elsewhere in this volume (347).

Hydrogen and Electron Transport

The final phase of respiration involves the transport of hydrogen (electrons) from the reduced pyridine nucleotides to oxygen, and it is here that over 90 per cent of the energy is trapped. There is good evidence that these reactions are also localized in the mitochondria. DPNH can be readily oxidized by mitochondrial preparations when oxygen is the only available electron acceptor, and in some cases the activity is confined largely to this cell fraction [Crane (70); Hackett (129)]. It is true that DPNH oxidase activity can be demonstrated in other cell fractions, but the soluble DPNH oxidase systems are probably artifacts of isolation which have nothing to do with normal respiration (43), and the activity in microsomes (230) and chloroplasts (163) is too small to play a significant role. This conclusion is supported by the wealth of evidence that the respiratory chain terminates with the mitochondrial cytochrome system (141, 331).

The relationship between the pathways of DPNH and TPNH oxidation has not been fully clarified. The available evidence indicates that the TPNH oxidase activity of plant mitochondria is very limited (129). In animal

mitochondria, TPNH may be oxidized slowly by an independent sequence of reactions (175), but in fragmented particles it is only oxidized in the presence of DPN (18, 197), presumably with the aid of a pyridine nucleotide transhydrogenase. This enzyme, which is present in plant (76) and animal (158) mitochondria, could play an important role in regulating the rate of TPNH oxidation, and thereby the intracellular level of TPN. In the presence of added electron acceptors, such as cytochrome-*c* (129, 230) or oxidized glutathione (225, 387), TPNH is rapidly oxidized by the mitochondria, but there is no evidence that these reactions are part of the main respiratory chain. The same is true for the active TPNH oxidases which have been demonstrated in soluble extracts (159, 228, 308). Using ATP, plant mitochondria can apparently convert DPN to TPN [Young & Conn (387)].

Enzymatic activities.—The DPNH oxidase activity of the mitochondria has been demonstrated with preparations from pea leaves [Smillie (326)], lupine cotyledons [Humphreys & Conn (160)], potato tubers [Hackett (127)], cauliflower buds [Crane (70)], skunk cabbage flowers [Hackett (129)], pepper fruits [Howard & Yamaguchi (156)] and beet roots [Honda, Robertson & Gregory (153)]. This activity can be completely blocked by cyanide and Antimycin A (70, 127, 129, 160). Since Antimycin acts specifically between cytochromes-*b* and *c*, and cyanide inhibits cytochrome-*c* oxidase, all of these components must be on the main oxidative pathway. The inhibitory effect of high phosphate concentrations (>0.02 *M*) may be due to changes in the particle structure or to the extraction of some cytochrome-*c* (70). With all of these preparations, the rate of DPNH oxidation can be increased severalfold by adding cytochrome-*c*. However, there is considerable evidence that the electron transport pathway which is activated under these conditions differs from the normal intramitochondrial path. For example, the mitochondrial DPNH-cytochrome-*c* reductase is only partially inhibited by Antimycin, with per cent inhibition values of 80 (160), 60 (129), 40 (127), 37 (70), 35 (230), and 30 (232) reported. Since cytochrome-*c* oxidase is present, this Antimycin-insensitive bypass opens up a more rapid "external" route from DPNH to oxygen. This pathway is apparently not available to succinate, for the mitochondrial succinic-cytochrome-*c* reductase is completely blocked by Antimycin (127, 230, 319); only with wheat root particles was a partial (50 per cent) inhibition of this reaction observed (232). The results suggest that the Antimycin-insensitive system which oxidizes DPNH is either on the "exterior" of the mitochondria or, more likely, on contaminating microsomes (see below). In either case, there is little reason to believe that this corresponds to a normal respiratory pathway. The rate of oxidation of TCA cycle acids is not generally increased by added cytochrome-*c*, unless the particles have been prepared under suboptimal conditions (37, 191, 252). Furthermore, the oxidation of these acids can be largely inhibited by Antimycin (124, 160).

The first step in the oxidation of DPNH is catalyzed by a flavoprotein, whose activity can be measured with a variety of electron acceptors. The TCA cycle acid dehydrogenases have frequently been assayed with the aid

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of dyes, and these reactions depend indirectly on the diaphorases. Using 2,6-dichlorophenol-indophenol, an active mitochondrial DPNH-diaphorase has been demonstrated, and in every case this activity exceeded that of the DPNH oxidase and DPNH-cytochrome-*c* reductase (70, 127, 230). As much as 60 per cent of the DPNH-diaphorase activity may be localized in the mitochondria [Crane (70)]. On the other hand, most of the activity may be found in the soluble fraction [e.g. Martin & Morton (230)], which is likely to contain a variety of DPNH-oxidizing systems of unknown function. Diaphorase activity has also been demonstrated in microsomes (230) and chloroplasts (160).

Some measure of the ability of mitochondria to transfer electrons to the cytochrome system has been gained by determining their DPNH-cytochrome-*c* reductase activity (64, 70, 127, 129, 153, 230, 232, 319, 328, 340). As pointed out above, only a part of this activity may depend on electron transport through the normal respiratory chain. By disintegrating the mitochondria, Davies (76) obtained a reductase in solution, and over a fortyfold purification the ratio of diaphorase to reductase remained constant. A flavoprotein (probably containing FAD) which acts as a DPN-specific reductase has been extracted from *Arum* spadix particles (167). An Antimycin-insensitive DPNH-cytochrome-*c* reductase system has been demonstrated in plant microsomes (70, 129, 134, 230, 232), but there is no reason to believe that it is normally concerned with electron transport to cytochrome-*c*, which is located in the mitochondria. The amount of reductase activity in the microsomal and mitochondrial fractions is roughly equal, and the specific activity in the microsomes may even be greater (70). DPNH- and TPNH-cytochrome-*c* reductases can be demonstrated in chloroplasts (227). Cytochrome-*c* can also be reduced by soluble components, such as chlorogenic acid, and these in turn may be reduced enzymatically by DPNH (320). The presence of this variety of cytochrome-*c* reducing systems makes it difficult to identify and evaluate the "purified" cytochrome-*c* reductases which have been prepared from extracts of whole tissues (94, 228, 308). The examination of their properties adds little to our knowledge of the respiratory chain itself.

All plant mitochondria possess an active cytochrome-*c* oxidase, the terminal enzyme of the respiratory chain (119, 126). Smith & Chance (331) dealt last year with recent studies on the oxidase and also pointed out the difficulties inherent in the assay systems. The inhibitory effects of high concentrations of cytochrome-*c* itself have now been shown with plant preparations (315). The report [Miller & Evans (250)] that relatively high salt concentrations promote the particulate cytochrome oxidase has been confirmed (153), but it seems likely that this is primarily a nonspecific physical effect, since the solubilized oxidase does not show this response [Miller, Evans & Sisler (251)]. By fragmenting the mitochondria with digitonin, Simon (315) has been able to increase the measured activity as much as fiftyfold. The optimal effect was observed after a very brief (10 sec.) treatment with a 2 per cent solution. This work adds considerable support to the argument that the low order of extractable oxidase reported for some plant tis-

sues is due to inadequate assay conditions rather than to a deficiency of the enzyme. There is probably enough cytochrome oxidase in all plant tissues to account for their respiratory rates, and in some cases there is a manyfold excess. The earlier conclusion that this enzyme is localized entirely in the mitochondria (119, 126) is supported by a recent study of cauliflower homogenates [Crane (70)], and the reports that it is present in the nuclear, plastid, and soluble fractions (297, 322, 323) can be attributed to differences in the techniques of isolation and assay employed. Attempts to purify the oxidase by ammonium sulfate fractionation of cholate extracts from roots have not given significant purifications (251, 319).

Spectroscopic observations.—The components of the respiratory chain exhibit characteristic spectroscopic properties, which are extremely useful for identification and analysis. The detailed reviews on plant cytochromes by Hartree (141) and Smith & Chance (331) should be consulted, and only the most recent work will be mentioned here. Many plant tissues show a characteristic broad absorption band around 550 to 560 mμ, and a similar band is seen in some hydrosulfite-reduced mitochondrial preparations (42, 319); other preparations show two bands in this region, at 553 and 562 mμ (143, 215, 233). Martin & Morton (233) conclude that the mitochondrial cytochrome-*b* (563 mμ) can be distinguished from the microsomal b_3 (560 mμ), and that both cytochromes-c_1 (554 mμ) and *c* (550 mμ) are present in the mitochondria; these conclusions require further support. Lundegårdh (215) has located the peaks in the difference spectrum of wheat root mitochondria at 428, 550 to 552, and 560–562 mμ, and he concludes that all the cytochromes which may be distinguished in intact wheat roots, including b_3, are present in this fraction. The difference spectra of deoxycholate-clarified cauliflower fractions give no indication of the existence of separate *b* hemes in the mitochondria and microsomes [Crane (70)]. It is clear that further work is needed on the localization and properties of the *b*- and *c*-type cytochromes. An apparently typical cytochrome-*c* has been removed from avocado mitochondria [Chow & Biale (64)] and crystallized from wheat germ [Hagihara *et al.* (137)]. The characteristic absorption bands of cytochrome-a-a_3 have been seen in all of the mitochondrial preparations mentioned above, but in some cases only at liquid nitrogen temperature [Bonner (42)]. In wheat root mitochondria, the molar ratios of b/a_3 ($\sim$4) and b/c ($\sim$2) are similar to those in the intact tissue (215). These particles also contain some dithionite-reducible cytochrome which does not appear under anaerobic conditions.

The respiratory components of Aroid spadix mitochondria, which in some cases contain an unusual cytochrome-*b*, have been studied further. On reduction with α-ketoglutarate, the difference spectrum of skunk cabbage mitochondria shows absorption bands due to cytochromes-a_3, a, b_7, c, and flavoprotein [Chance & Hackett (59)]. The absorbency changes indicated relatively high concentrations of both the *b*- and *c*-type cytochromes, relative to a, and also a high a_3/a ratio. Whereas Bendall & Hill (31) described two *b* components in *Arum* mitochondria, with bands at 560 (b_7) and 563 mμ (*b*), the b_7-like component of skunk cabbage particles was displaced to 558 mμ

and no evidence was obtained for a typical *b* component. On the other hand, a component, with a similar α absorption maximum and a low γ/α band ratio ($\sim$4), which could only be reduced by dithionite was identified. In liquid nitrogen the α band of b_7 apparently splits into two peaks, at 556 and 558 mμ. This agrees with the observations of Yocum & Bonner (384) in this same material, although the behavior of their 552 mμ band shows some differences. The role of the b_7 component will be considered in connection with inhibitor-resistant respiration.

The respiratory chain.—The enzymatic and spectroscopic studies of isolated plant mitochondria support the following general scheme of electron transport:

$$\text{DPN} \rightarrow \text{FP} \rightarrow \text{Cyt-}b \rightarrow \text{Cyt-}c \rightarrow \text{Cyt-}a\text{-}a_3 \rightarrow \text{O}_2.$$

This is in agreement with observations which have been made on intact tissues (331). Where calculated, the turnover numbers for the plant cytochromes have been similar to those reported for other tissues [Lundegårdh (211); Yocum & Hackett (385)]. Recent experiments with skunk cabbage mitochondria [Chance & Hackett (59)] have shown that the steady-state oxidation-reduction levels of the components during α-ketoglutarate oxidation—15, 25, 10, 5, and 10 per cent reduction for PN, FP, cytochromes-*b*, *c*, and *a* respectively—correspond roughly to those found in "active state" animal mitochondria (61). The possible roles of other factors, such as vitamins E and K, in the electron transport systems of plants have not yet been critically examined. Crane & Lester (71) have recently reported that plant tissues contain a lipide-soluble quinone, with an absorption maximum at 254 mμ (Q_{254}), which is analogous to the Q_{275} or mitoquinone implicated in animal respiratory systems. In spite of the general similarities between the plant and animal respiratory chains, there are also indications of real differences. There is frequently less cytochrome-*a* in plants, relative to the amounts of *b* and *c*, and the ratio of cytochrome oxidase activity to 605 mμ absorption may be unusually high (59). In lower plants, the cytochrome-*a* and a_3 absorption bands may be scarcely distinguishable [Chance & Sager (60)]. There is not yet any clear-cut evidence for the presence and activity of cytochrome-c_1 in plants. The exact nature of the mitochondrial cytochrome-*b* component(s) and its relationship to b_3, b_7, and the dithionite-reducible material remain to be clarified. Lundegårdh (213) has reported that cytochrome-*b* is reduced more slowly in roots than in yeast, and other indications that the higher plant respiratory chain may be modified in the *b*-region will be considered below.

Alternate pathways.—A variety of other oxidases and systems which can transport hydrogen from PN to oxygen are demonstrable in plant extracts, but there is still no evidence that these noncytochrome pathways are important in respiration (43). The recently described soluble [Romberger (290)] and particulate [Stern & Johnston (340)] DPNH oxidases, which are readily inhibited by cyanide and ascorbic acid, resemble the wheat germ

TPNH oxidase system which was shown earlier to involve peroxidase, Mn^{++}, and a cofactor (67, 159). Akazawa & Conn (5) have now reconstructed a similar system using crystalline peroxidase and various phenols. They suggest that the overall reaction involves a peroxidase-catalyzed oxidation of the phenol by molecular oxygen, followed by the transfer of hydrogen from DPNH to the oxidation product. Since there is little evidence to support the role of peroxidase as a terminal oxidase, Yamazaki, Fujinaga & Takehara (380) have suggested that it may have some intermediate hydrogen-transfer role in plant respiration.

There is general agreement that the copper-containing oxidases are not major respiratory enzymes; nevertheless, some still claim an important function for the ascorbic acid system, and the review by Mapson (217) should be consulted for an evaluation of the recent evidence. Papers continue to appear, from the Russian workers [e.g., (248)] and others (236), in which the mere presence of an active oxidase is considered sufficient basis for suggesting its role as a terminal respiratory enzyme, but this conclusion is entirely unacceptable in the absence of confirmation at the tissue level. An intermediate role for ascorbic acid is suggested by Arrigoni, Rossi & Marrè (12), who have shown that the DPNH oxidase of pea internode mitochondria is promoted by ascorbic, but not by dehydroascorbic, acid. They suggest that a "monodehydroascorbic" intermediate is involved in hydrogen transport, possibly to the cytochromes. This system is clearly different from the TPN-glutathione-dehydroascorbic acid system that has also been demonstrated in plant mitochondria [Marrè & Laudi (225); Young & Conn (387)]. DPNH can be oxidized by tobacco root extracts via chlorogenic acid and polyphenol oxidase [Sisler & Evans (320)]. Protocatechuic acid can be oxidized by tissue slices via polyphenol oxidase [Middleton (247)]. In none of the above-mentioned cases have the reactions been shown to be part of the main respiratory pathway.

The flavin oxidases do not in general show the appropriate inhibitor and oxygen affinity characteristics to be able to account for much plant respiration. Nevertheless, the possible participation of glycolic acid oxidase, particularly in green tissues at high light intensity, is still being considered [Delavan & Benson (80); Zelitch (391)]. Although roots do not contain this enzyme, they, like other tissues, form it when exposed to light [Mothes & Wagner (256)]. Zelitch (390) has synthesized inhibitors of glycolic oxidase which may be useful in testing its possible role in respiration. The preparation and properties of crystalline glycolic acid oxidase from spinach have been described [Frigerio & Harbury (107)]. A reduced pyridine nucleotide oxidase system which apparently utilizes a flavoprotein with an unusually high oxygen affinity has been demonstrated in acetone powder extracts of pea leaves [Marrè & Servettaz (226)]. A flavin oxidase which is involved in bacterial respiration has a relatively high oxygen affinity, but this is still considerably lower than that of cytochrome oxidase [Niederpruem & Hackett (268)]. Under conditions where there is an adequate rate of oxygen supply, plant tissues, includ-

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ing those with a cyanide-resistant respiration (385), respire with a high affinity for oxygen, and this fact argues against the role of flavins as terminal oxidases.

Although lipoxidase may catalyze a considerable portion of the oxygen uptake by plant homogenates [Fritz & Beevers (108)], it does not act as part of a DPNH oxidase system [Siddiqi & Tappel (313)]. A recent study on O^{18} incorporation, from O^{18}-enriched oxygen, by corn seedlings indicates that lipoxidase could only account for 0.4 per cent of the respiration [Fritz *et al.* (109)].

Cyanide- and Carbon Monoxide-Resistant Respiration

The major argument which has been used against the participation of a typical cytochrome chain rests on the fact that the respiration of many plant tissues is not easily inhibited by cyanide and CO; the evidence has been described in detail in several recent reviews (141, 165, 331). It should be pointed out that in many of these cases the oxygen uptake is actually increased by the "inhibitor," while various energy-requiring processes, such as water uptake (135), ion uptake (140), and photosynthesis (389), are markedly inhibited. These facts indicate that the tissues are not entirely "insensitive" and that the inhibitors are actually combining with some cell component. It must also be borne in mind that cytochrome oxidase has been demonstrated in all the inhibitor-resistant tissues, including the reportedly exceptional apple fruit (199, 261, 297). This raises the possibility that the atypical response to inhibitors is the result of a quantitative, rather than a qualitative, difference in the respiratory chain (148). For example, the presence of an excess of cytochrome oxidase relative to the rate-limiting step might make it possible to inhibit a large fraction of this enzyme without affecting the overall rate of electron transfer. Evidence has long been available that this is the situation in certain resistant microorganisms which become sensitive to inhibitors when the respiratory rate is increased by added substrates (92, 275). Lundegårdh (214) has shown that yeast respiration may be inhibited as little as 12 per cent when 82 per cent of the cytochrome-a_3 is combined with cyanide, and somewhat similar data were obtained earlier with roots (210). Ducet & Rosenberg (84) were able to demonstrate a CO-inhibition of leaf respiration by growing plants without iron, and they explained the normal CO-insensitivity in terms of this excess-oxidase hypothesis. Whatever may be the explanation, it is clear that failure to observe respiratory inhibition does not exclude the cytochrome system, and other spectroscopic and enzymatic evidence must be obtained before concluding that an alternate pathway is operating.

Aroid spadix.—Some, but not all (143), Aroid spadices have a very rapid, cyanide-resistant respiration, and this has attracted special attention in recent years. Independent work on English [Bendall & Hill (31)] and American [Yocum & Hackett (385)] Aroids showed spectroscopically that the cytochrome system participates in the respiration of these tissues. Since the cytochrome-*b* component (α peak at 560 mμ) remained oxidized in the presence

of sufficient cyanide to cause a large reduction of cytochromes-*a* and *c*, both groups suggested that the apparently autoxidizable *b* component, called b_7 (31), might serve as an alternate, inhibitor-resistant oxidase in this and other plant tissues. (A similar suggestion was made by Lundegårdh (212) on the basis of his observation of the partial oxidation of cytochromes-*b* and *c* in wheat roots exposed to cyanide.) The demonstration that the cytochromes, including the unusual *b* component, are localized in the mitochondria isolated from the Aroid spadix suggests that the respiratory system is on these particles [Bendall & Hill (31); Bonner & Yocum (46); Hackett & Haas (133)]. This conclusion is supported by the reports that the TCA cycle acid oxidations by spadix mitochondria are relatively resistant to 10^{-3} *M* cyanide [James & Elliott (169); Simon (314); Hackett (129)]. In spite of this, the DPNH oxidase could be inhibited 80 per cent by either 10^{-3} *M* cyanide or by Antimycin, and it was not possible to demonstrate a cyanide-resistant mitochondrial respiratory chain (129). Since DPNH is oxidized at a several-fold greater rate than the organic acids, the results could be explained if the degree of inhibition depends on the extent of activation of the respiratory chain. In other words, the lack of inhibition of the TCA cycle oxidations (and tissue respiration) could be due to a lower degree of activity in the electron transfer system. It is significant that there is a very active cytochrome-*c* oxidase on the spadix mitochondria and this is completely inhibited by cyanide (129, 314, 385) and azide (46).

In order to test critically the cytochrome-*b* oxidase hypothesis, it is necessary to completely eliminate or inhibit the *c*-*a* system. Although Bendall & Hill (31) were unable to detect spectroscopically any oxidized cytochrome-*a* or *c* in the aerobic, cyanide-treated *Arum* mitochondria, spectrophotometric measurements have indicated that 50 per cent (385) and 20 per cent (133) of the skunk cabbage cytochromes-*c* and *a* remain oxidized in 0.2 m*M* and 1 m*M* cyanide, respectively. The extent of reduction of the components in skunk cabbage mitochondria has recently been measured under a variety of conditions, using more sensitive techniques [Chance & Hackett (59)]. When α-ketoglutarate was the substrate, 10 m*M* azide inhibited the oxygen uptake only 14 per cent and the respiratory components were reduced 15 per cent (PN), 45 per cent (FP), 30 per cent (b_7), 80 per cent (*c*), and 86 per cent (*a*). In the presence of the more rapidly oxidized DPNH, up to 95 per cent reduction of cytochromes-*c* and *a* was observed, but neither with cyanide nor azide was it possible to reduce them completely. This finding, together with the fact that there is an active cytochrome oxidase with a high turnover number, indicates that the excess hypothesis cannot yet be completely eliminated. The ability of the electron-transfer components to show large changes in their steady states, in portions of the system where their concentrations are not rate-limiting, without appreciably changing the total transfer rate has been termed the "cushioning effect" [Chance (58)]. The skunk cabbage mitochondria, in which the respiratory components are only slightly reduced by α-ketoglutarate, are appropriately proportioned for such an effect. The rate-limiting step in this chain may be at the level of intramitochondrial

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DPN itself. It is interesting that the particles may be rendered azide-sensitive by altering the pH and osmotic concentration of the isolating medium [Bonner (44)].

Some insight into the possible role of the b_7-type cytochrome of skunk cabbage particles is provided by the finding that it can be reduced by Antimycin A and 2-*n*-heptyl-4-hydroxyquinoline, which act specifically between cytochromes-*b* and *c* [Chance & Hackett (59); Yocum & Bonner (384)]. It has also been shown spectrophotometrically that the mitochrondial cytochrome-*c* can act as an oxidant for b_7 (59). These results suggest an electron transfer series of $b_7 \rightarrow c \rightarrow a\text{–}a_3 \rightarrow O_2$. Although the kinetics of oxidation and reduction of the b_7 component are consistent with this sequence, the relatively slow rate of its reduction in the aerobic-anaerobic transition suggests that the respiratory chain may be branched in the *b*-region. If there is an alternate, cyanide-resistant pathway from b_7 to oxygen, due possibly to its autoxidation, it can apparently be blocked by Antimycin. Although the *b*-type cytochromes, such as those in microsomes (229), are known to be autoxidizable, this reaction has not yet been shown to be sufficiently rapid to be important in respiration. The same is true for the b_7 solubilized from spadix mitochondria [Bonner & Smith (45)]. The suggestion that the respiratory chain in some microorganisms terminates with a cytochrome-*b* (39) has not been critically tested.

Potato slices.—A rapid, inhibitor-resistant respiration develops during a one-day, aerobic incubation of potato tuber slices, and this presents an ideal opportunity for studying the transformation of a "typical" respiratory system into an "atypical" one [Thimann, Yocum & Hackett (357)]. The mitochondria isolated from the two types of tissue (i.e., fresh and aged slices) show similar rates of TCA cycle acid oxidation, but the aged-tissue particles are considerably less sensitive to cyanide [Hackett & Haas (134)]. There is a severalfold increase in the mitochondrial DPNH oxidase, diaphorase, and DPNH-cytochrome-*c* reductase activities, but very little change in the succinic-cytochrome-*c* reductase and cytochrome-*c* oxidase. The rapid DPNH oxidase remains sensitive to cyanide, azide, and Antimycin A. Difference spectra (reduced with DPNH-oxidized) of the mitochondrial suspensions show a marked increase in the amount of cytochrome-*b* relative to $a\text{–}a_3$ during the incubation. The evidence suggests that there is a transformation in the mitochondrial respiratory chain, particularly in the cytochrome-*b* region, and the properties of the new system resemble those encountered in Aroid spadix mitochondria. This adds support to the view that the unique characteristic of inhibitor-resistant tissues is their high concentration of cytochrome-*b*, rather than cytochrome oxidase. If the oxidase is always in excess, this situation may make it possible to maintain the usual respiratory components in a more oxidized or "cushioned" steady-state. Alternatively, it might be argued that the increased amount of *b*-type cytochrome acts as the inhibitor-resistant oxidase.

Whatever may be the nature of this change in potato slices, it is closely related to a general increase in metabolism and synthesis [Mulder (260)],

and it can be prevented by cyanide, anaerobiosis [Hackett (128)], and chloramphenicol [Calo, Marks & Varner (55)]. This transformation or synthesis within the cytochrome system may be similar to that which accounts for the reversal of cyanide inhibition in wheat roots [Lundegårdh (211)]. It is well known that the cytochrome system in some microorganisms is readily modified by changes in such environmental conditions as the oxygen tension (255); in animal cells, this system can undergo marked transformations during development (309). Similar environmental and developmental factors may well modify the higher plant cytochrome system (148).

Barley roots.—James' claim that the cytochrome system in seven-day barley root tips is inadequate to account for the respiration was carefully reviewed in the last two volumes of this series (43, 217). In a recent paper, James & Ward (170) have shown that the characteristic decrease in CO-inhibition and the increase in DIECA-sensitivity during development can be demonstrated with barley roots but not with wheat roots. However, Eliasson & Mathiesen (91) report that the sensitivity of wheat root respiration to azide and cyanide does decrease with distance from the tip. Stolwijk & Thimann (343) were able to show a light-reversible CO-inhibition of barley root respiration, and Lubell & Bonner (207) could find no correlation between the age of the barley roots and the CO-sensitivity of respiration. Tissue-level studies of the effects of oxygen tension on barley roots suggest that the terminal oxidase has a high affinity for oxygen, comparable to that of cytochrome oxidase [Hopkins (154); Lubell & Bonner (207)]. Mitochondria prepared from barley roots at all stages of development show cytochrome oxidase activity, and it seems likely that the change in response to CO is due to quantitative changes within the usual respiratory chain. Although the "DIECA effect" has not been satisfactorily explained, the available evidence does not compel us to postulate an entirely different respiratory pathway (e.g., involving ascorbic oxidase) in barley roots (152).

Effects on phosphorylation.—Any general explanation of the resistance of respiration to cyanide and CO must also be able to account for the frequently observed stimulations of oxygen uptake and inhibitions of energy-requiring process. This type of effect could be due to an "uncoupling" of respiration from oxidative phosphorylation, as suggested earlier (357). Such an explanation implies that even in resistant tissues the phosphorylation is linked to electron transfer in the cytochrome system. During the development of the inhibitor-resistant respiration in potato slices, there is an exponential increase in their capacity to take up P^{32} [Sharpensteen (310)] and to incorporate it into organic compounds [Loughman (204)]; it is significant that this P^{32}-uptake can be light-reversibly inhibited by CO, indicating that it is dependent on the cytochrome system [Griffiths & Hackett (122)]. Ducet & Rosenberg (85) have recently shown that the CO-induced increase of oxygen uptake and aerobic glycolysis in old tobacco leaves are associated with a decrease in acid-soluble organic-phosphate compounds and an increase in inorganic phosphate, as would be predicted if CO is uncoupling phosphorylation. In *Chlorella*, the cyanide-induced increase in oxygen up-

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take is accompanied by a decrease in the intracellular ATP level [Syrett (351)]. The ability of cyanide to act as an uncoupling agent has now been demonstrated directly [Hackett & Haas (133, 134)]: with mitochondria isolated from the skunk cabbage spadix and aged potato slices, low concentrations of cyanide can inhibit phosphorylation without affecting the rate of oxygen uptake, i.e. it can decrease the P/O ratio (phosphate esterified /oxygen consumed). In both cases, the high P/O values in the absence of inhibitor suggest that electron transfer proceeds through a typical respiratory chain. Assuming then that these inhibitors can act as uncoupling agents, what type of effect is it that permits electron transfer to oxygen but not the coupled phosphorylation? One possible explanation is that the inhibitor blocks the normal respiratory chain and diverts electrons to a resistant, nonphosphorylating pathway (e.g., cytochrome-$b \rightarrow O_2$) which may not play an appreciable role in the absence of inhibitor. Another possibility is that phosphorylation is more sensitive to the steady-state oxidation reduction levels of the respiratory components, so that increased reduction may block phosphorylation even though electron transfer through the normal chain is not inhibited. It is also possible that the inhibitors may act on some site, other than the iron of cytochrome oxidase, which is specifically involved in phosphorylation.

Phosphorylation

The energy-supplying function of respiration is realized with the formation of high-energy phosphate bonds, which takes place chiefly during the transfer of electrons to oxygen. Oxidative phosphorylation has now been successfully demonstrated with particles prepared from a wide variety of plant tissues, including mung bean (110, 253, 280) and soybean (350) hypocotyls; pea stems (326); pea (87), corn (209), wheat (87), and barley (87) roots; pea (87, 326), spinach (9, 272), and cabbage (106) leaves; peanut (182, 240), lupine (69), soybean (157), and corn (138) cotyledons; castor bean endosperm (4); cauliflower buds (110, 191); skunk cabbage flowers (133); avocado (37, 289) and pepper (156) fruits; artichoke (41) and white potato (134) tubers, and sweet potato storage roots (6, 201).

Contrary to a recent statement that P/O ratios higher than one are rather rare (167), the majority of reported values indicate that several high-energy bonds are formed, per pair of hydrogens transferred to oxygen, during the oxidation of TCA cycle acids. Values approaching the theoretical maxima for the P/O ratio, i.e., greater than 3.0 with α-ketoglutarate (4, 69, 106, 133, 201), greater than 2.0 with citrate (4, 326), greater than 1.0 with succinate (6, 69, 86, 87, 106, 110, 191, 326), have frequently been obtained. There is little doubt that the energy-trapping mechanisms in plants can be as efficient as they are in animals. In addition to the usual adenylate and Mg^{++} requirements for phosphorylation, hexokinase, fluoride, and malonate have increased the measured P/O values in some cases. A number of constituents which are commonly encountered in plant homogenates, including Ca^{++} (234), chlorogenic acid (6), catechol (201), and microsomes (182), can in-

hibit phosphorylation. These factors, as well as differences in mitochondrial stability (110) and ATPase activity (4, 86) may account for some of the low P/O values reported. The reaction products of oxidative phosphorylation have been identified, using P^{32}, as ATP and the other adenosine phosphates (69, 240, 253); when glucose is included as a trapping agent, labelled glucose-6-phosphate is also formed (69).

Mechanisms.—The P/O values quoted above indicate that there are three phosphorylative steps between PN and oxygen [cf. (196)]. The only reported attempts to use extramitochondrial DPNH and TPNH as substrates in phosphorylation experiments gave P/O values of less than one [Smillie (326)]. The fact that cyanide (110, 133) and Antimycin (182) inhibit phosphorylation supports the assumption that a typical respiratory chain is involved. Relatively little has been done towards the specific localization of energy-conservation sites in plant mitochondria. Using mung bean particles, Fritz & Naylor (110) were able to localize one phosphorylative step between cytochrome-*c* and oxygen, and another between succinate and the point at which ferricyanide intercepts the respiratory chain (presumably near cytochrome-*c*). In cauliflower mitochondria, the second of these steps was very labile. Kmetec & Newcomb (182) observed phosphorylation during the oxidation of cytochrome-*c* but were unable to detect it in the preceding steps. These results may be compared with the observation that the step between cytochromes-*b* and *c* is the most labile in animal mitochondrial fragments (197). Although the spectrophotometric method of site localization (61) has not yet been used with plant mitochondria, an interaction between ADP and cytochrome-*b* was observed [Chance & Hackett (59)]. The cytochrome-*b* region may play a special role in phosphorylation; a close correlation between the amount of *b* and the magnitude of the salt respiration has been noted [Lundegårdh (211)]. The attempt to localize sites of phosphorylation by a kinetic analysis of phosphate uptake by intact barley roots treated with inhibitors and substrates [Hagen, Leggett & Jackson (136)] must be considered speculative in the absence of simultaneous measurements of the electron flux and steady-state levels of the respiratory components. A final piece of evidence which indicates that the phosphorylations by plant mitochondria are linked to the activity of the respiratory chain is the fact that they can be uncoupled by DNP, which blocks all but the substrate-level step (4, 69, 86, 133, 191, 201, 240). This action is sometimes 4, 69, 201), but not always, accompanied by an increased oxygen uptake. The relative effectiveness of a number of nitro- and halo-phenols has been examined [Gaur & Beevers (111)]. An interesting, and as yet unexplained, case of DNP-resistant phosphorylation has been demonstrated with ripe avocado mitochondria [Romani & Biale (289)]. It is significant that in those cases where the mitochondrial oxidations do not necessarily involve the cytochrome system, as when chlorogenic acid, ascorbate, and glutathione are the substrates, there is no evidence of any coupled phosphorylation [Akazawa & Uritani (6); Young & Conn (387)].

The mechanism which couples electron transfer to the synthesis of ATP

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almost certainly involves several unknown intermediate steps. The uncoupling action of DNP can be attributed to the decomposition of some energy-rich intermediate. The mitochondrial ATPase activity (126) probably results from a reversal of the last step in the coupling mechanism. Forti (100) has recently studied the ATPase of pea stem mitochondria; the activity was greatly increased by low concentrations of phosphate, while Ca^{++}, DNP, and aging gave smaller promotions. The finding that beryllium is a much more effective ATPase inhibitor than fluoride suggests that it might be useful in P/O determinations. He obtained evidence that only the terminal phosphate of ATP is hydrolyzed in this reaction; ADP is an effective inhibitor. Studies on the mechanism of hydrolysis of ADP and ATP by purified potato apyrase have shown that the cleavage occurs between O and the terminal P in both cases [Cohn & Meek (66); Lee, Krezanoski & Eiler (195)]. Adenylic kinase has been demonstrated in plant mitochondria (110, 239) and some properties of the solubilized enzyme investigated [Davies (76); Mazelis (239)]. By regulating the balance among the adenine nucleotides, this enzyme may control a variety of metabolic pathways. The enzyme which phosphorylates ADP during the breakdown of succinyl-CoA has been purified from spinach leaves [Kaufman & Alivisatos (177)].

Regulation and storage.—The regulation of plant respiration is intimately related to the formation and utilization of high-energy phosphate bonds (19, 192). The most generally accepted hypothesis is that the intracellular level of phosphate acceptor (ADP) controls the respiratory rate. In an analysis of the Pasteur effect, Rowan, Seamen & Turner (294) found a definite increase in the ADP/ATP ratio when pea seeds were transferred from air to nitrogen. On the other hand, in yeast there is evidence that the Pasteur effect may not be directly correlated with the ADP and ATP levels (194). In *Chlorella*, Syrett (351) has shown that increases in respiration induced by DNP, glucose, and NH_4^+ are accompanied by rapid decreases in the ATP level, but there was not a direct proportionality between these events. In all of these cases, it must be remembered that the determining factors are probably the intramitochondrial nucleotides, which make up only a part of the total cell concentration and may not be easily influenced by the extramitochondrial nucleotides (284). The short-term incorporation of phosphate into the nucleotides of intact barley plants has been followed [Loughman & Russell (206)], using methods recently developed for this purpose [Loughman & Martin (205)].

Several recent papers have dealt with methods for the separation and identification of the acid-soluble phosphates, including the nucleotides, in plant tissues (83, 292, 306). Procedures for the precipitation of adenosine phosphates have been compared (120). Practically nothing is known about the storage of energy-rich phosphates in higher plants. The demonstrations of reversible, enzymatic phosphate transfer from inorganic polyphosphates to ADP by bacterial systems (19, 375) indicate that these compounds, which are known to be present in plants, may serve as energy reservoirs. A recent study has established the widespread occurrence of polyphosphates in

lower plants, but they were not present in the higher plants examined [Keck & Stich (178)]. In seeds, phosphate is commonly accumulated as phytin and the possibility that this plays a role in energy storage should perhaps be considered. ATP can act as the donor in the phosphorylation of meso-inositol (150), and phosphate bonds in the hexaphosphoric acid derivative of inositol, i.e., phytic acid, should be relatively unstable. Higher plant enzymes which hydrolyze polyphosphates (279) and phytin (8, 237) have recently been studied.

Respiratory Mechanisms in the Light

It is not yet possible to give a simple, definitive answer to the important question of whether light has any quantitative or qualitative effect on the respiration of photosynthesizing tissues [Whittingham (374)]. The widely-quoted isotope studies of Brown (49) showed that in most cases the respiratory rate is the same in darkness and in light. However, there were cases of both photoinhibition and photostimulation, and the method used is open to the criticism that it does not measure the consumption of unlabelled O_2 which is produced photosynthetically within the cells. In the work of Bassham, Shibata & Calvin (22) it was assumed that there is an "extra respiration" which increases with the light intensity. The recent demonstration that CO_2 is given off by leaves in the light has raised the possibility of a light-induced "additional respiration" [Krotkov, Runeckles & Thimann (186)]. Warburg (372) has recently reviewed his argument that O_2 is necessary for a respiratory back-reaction involved in photosynthesis, even though severe restrictions of the O_2 supply are apparently without effect on photosynthesis.

If there is an "extra respiration" in the light, the question arises as to whether it takes place in the chloroplasts or in the mitochondria, the normal dark respiratory centers. In the latter case, the effect would have to be exerted at some distance from the primary photochemical events. Isolated chloroplasts show no endogenous oxygen consumption and are unable to oxidize the TCA cycle acids [Arnon, Allen & Whatley (9); James & Das (168)], although the presence of mitochondrial fragments in the chloroplast preparation may obscure this conclusion (10, 272). The possibility that light may activate a different respiratory system has been raised by the demonstrations of light-stimulated glycolic acid oxidation [Delavan & Benson (80)] and cytochrome-*c* photooxidase [Nieman & Vennesland (271)] in chloroplasts. Although isolated chloroplasts can carry out a rapid photochemical reduction of O_2 (Mehler reaction), the reaction apparently plays no role *in vivo* [Brown & Good (50)]. Since there is no clear evidence that the chloroplasts have a respiratory role, the "extra respiration," if there is any, may be the result of secondary interactions with the mitochondria.

On the basis of his $C^{14}O_2$ studies with algae, Calvin (56) suggested that the operation of the TCA cycle is slowed down by light. This hypothesis is not supported by recent studies with wheat [Bidwell, Krotkov & Reed (38)], barley [Naylor & Tolbert (263)], and *Bryophyllum* [Moyse & Jolchine (259)]

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leaves, in which it has been shown that the cycle is at least as active in the light as in the dark. The metabolism of labelled glutamate and glutamine via the cycle was actually accelerated by the light. Milhaud, Benson & Calvin (249) have also found that the metabolism of exogenous pyruvate by *Scenedesmus* is not slowed down by light. The various observations suggest that there is a competition for those intermediates, such as phosphoglyceric acid which can be either oxidized in the dark or reduced in the light. During photosynthesis, the newly-fixed CO_2 does not readily enter the TCA cycle, but substrates for the cycle oxidations are readily supplied from endogenous pools. The apparent light-induced increase in the malonate sensitivity of leaf respiration supports the conclusion that the TCA cycle is operating [Farkas Konrad, & Király (96)].

Other mechanisms of interaction with the mitochondria are suggested by the fact that photosynthesizing chloroplasts generate both reducing power and ATP [Arnon, Whatley & Allen (11)]. Thus, a marked change in the intracellular ADP/ATP ratio in the light might be expected to influence the respiratory rate. In a recent study with *Elodea*, Simonis & Weichart (318) found that the amount of ADP+ATP in the light may be either less than or greater than the amount in the dark, depending on the length of the light period. Although there are transient changes in ADP and ATP when *Scenedesmus* is illuminated, the steady-state levels are apparently not much different in the dark and in the light [Bradley (47)]. These interrelations are certain to be complex in the multicompartmental cell system. The fate of the photosynthetically-generated ATP has not been completely mapped out, but the structure of the chloroplast, with its many internal discs, would seem to favor its utilization near the sites of production. The possibility that it may be used within the chloroplast for processes other than carbohydrate synthesis is supported by the fact that chloroplasts can synthesize proteins, and this is stimulated by light [DeDeken-Grenson (77); Stephenson, Thimann & Zamecnik (339); Sissakian (321)].

The question remains as to whether ATP can be exported from the chloroplasts to underwrite the energy requirements of the cell, which are otherwise met by ATP generated in the mitochondria. The rapid rate of photosynthetic phosphorylation and its possible evolutionary significance (11) make this hypothesis worth examining. It has long been known that light can affect the movement of many electrolytes and nonelectrolytes into plant cells (48). Using inhibitors, van Lookern-Campagne (364) has shown that active chloride absorption by *Vallisneria* leaves can be supported either by respiration or by a light-driven reaction which does not require the assimilation of carbon; the simplest explanation is that the necessary ATP may be supplied either by oxidative or photosynthetic phosphorylation. Similarly, the energy-requiring assimilation of exogenous glucose may be linked to photosynthetically-supplied ATP, in which case the glucose uptake and CO_2 assimilation may compete for ATP (173, 304, 316). The reverse situation, in which respiratory ATP would contribute to the photosynthetic reactions, should also be considered. The report that light may affect the phosphate

turnover even in chlorophyll-free tissues, such as barley roots [Simonis & Ehrenburg (317)], indicates the need for caution in interpreting all of these results.

Physiological Correlations

In recent years, a wide variety of physiological processes have been analyzed in relation to the underlying respiratory events [Laties (192)]. It is to be hoped that continued studies along these lines will help to bridge the gap between the respiratory mechanisms and the complex activities of the plant.

Nitrogen and sulfur assimilation.—It is well known that the respiration of nitrogen-deficient cells can be stimulated by the addition of ammonium or nitrate salts. The assimilation of ammonia by barley roots is accompanied by a synthesis of glutamine, so that the increase in respiratory rate may result from the rapid utilization of ATP in this synthesis [Yemm & Willis (383)]. Folkes & Yemm (99) have recently emphasized the close correlation between the rates of respiration and protein synthesis in barley endosperm. With nitrate, a step-wise reduction must precede the assimilation, and there is good evidence that respiratory substrates supply the necessary reducing power (334). Although it is known that pyridine nucleotides are involved and that nitrate (or nitrite) can compete with O_2 as terminal electron acceptors, the pathway of this electron transport and its relation to the respiratory chain are not known. A soluble molybdoflavoprotein which can act as a nitrate reductase has been isolated. There is also evidence for the participation of cytochromes in the overall reduction and some of the steps may involve phosphorylations [Nicholas (267)]. If there is a common pathway for the electrons going to O_2, on the one hand, and to nitrate or nitrite, on the other, the branch-point probably occurs below the level of cytochrome-*b*, since 2-*n*-heptyl-4-hydroxyquinoline [Medina & Nicholas (245)] and Antimycin [Cheniae & Evans (63)] can block nitrate reduction. This suggests that the mitochondrial respiratory chain may be important, but the question of the normal intracellular localization of this reduction has not been settled; both particulate (81) and soluble (88) fractions have been reported to be effective.

A recent report indicates that TPN and ATP are required for the reduction of sulfate [Wilson & Bandurski (377)]. It will be of interest to know whether cytochromes are also involved, as they are in bacteria, and to relate the reaction to the respiratory chain. Plant mitochondria are able to oxidize sulfite to sulfate [Tager & Rautanen (366)].

Development.—During germination, the respiratory rate generally rises to a maximum after a few days and then declines (164). Mitochondria have now been isolated from a wide variety of germinating seeds and in most cases their ability to oxidize the TCA cycle acids changes in parallel with the respiration (4, 30, 138, 157, 282, 336, 337, 378). The increase in oxidative activity during the first few days is apparently due to changes in both the mitochondrial mass and the specific activity [Akazawa & Beevers (4)]. During

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this same period, there may be a decrease in the phosphorylative efficiency (P/O) which is related to a concurrent development of ATPase activity. Some germinating seeds contain soluble factors which can either inhibit [Beevers & Walker (30)] or promote [Stanley & Conn (337)] the oxidative activity of the mitochondria. Tissue level studies have also provided evidence for the activity of the TCA cycle during germination [Krupka & Towers (187); Haber & Tolbert (125)]. Many individual respiratory enzymes and cofactors have been assayed during germination and increases in the following are of interest: DPN (35), FMN and FAD (34), malic and succinic dehydrogenases (238, 358), cytochrome oxidase (142, 358), phosphatase (142), and ATPase (386).

Respiratory gradients in roots have been studied in many laboratories and recent reports have confirmed the fact that the respiratory rate, based on the amount of protein or acid-insoluble phosphorus, is lower in the root tip than in the zone of cell elongation [Betz (32); Eliasson (90); Jensen (171); Honda (151)]. Lund, Vatter & Hanson (209) have shown that this can be explained, at least in part, by the increase in mitochondrial mass and oxidative activity, per unit of protein, along the root. Relatively little is known about the shoot apex, but it has recently been shown that the respiratory rate is higher in the corpus than in the surrounding tunica of the *Lupinus albus* apex [Sunderland, Heyes & Brown (348)]. Cambial activity has been correlated with high dehydrogenase activity [Gerola & Barbesino (112)]. Mikhlin & Mutuskin (248) claim that there is a differentiation of oxidizing enzymes during plant development, but their argument that the various noncytochrome oxidases function in respiration lacks experimental support.

Fruit maturation.—The respiratory changes accompanying fruit maturation, particularly during the climacteric, have attracted considerable interest. Several lines of recent evidence suggest that phosphorylation is not impaired during the ripening process: there is no decrease in the total amount of high-energy phosphate compounds [Rowan, Robertson & Pratt (293)], P^{32} continues to be incorporated into organic compounds [Marks, Bernlohr & Varner (219)], and the mitochondria isolated at various stages carry on an efficient oxidative phosphorylation [Romani & Biale (289)]. These findings suggest that the observed changes in the respiratory rate are probably more closely linked to the level of synthetic activity in the tissues than to the action of some "uncoupling agent." With pea fruits, Rowan & Turner (295) were unable to discover any simple relationship between the intracellular ADP/ATP ratio and the respiratory rate. During the maturation of pepper fruits, there is a striking increase in the oxidative activity of the isolated mitochondria, and evidence has been obtained that this is the result of some change in the respiratory chain [Howard & Yamaguchi (155, 156)]. During the ripening of avocados, the ability of the supernatant fraction to stimulate mitochondrial oxidations increases markedly (289). Tager (354) and Tager & Biale (355) have suggested that when bananas ripen there is a shift from the pentose cycle to the EMP pathway of carbohydrate breakdown. An active malate decarboxylation has been correlated with the apple fruit climac-

teric (265). The production of ethylene by fruits is intimately linked to respiration, but it is probably the result, rather than the cause, of the ripening process (36, 53, 379).

Hormone action.—The mechanism of auxin action continues to be examined from the point of view of its effect on the respiratory system [Audus (13)]. The hypothesis that auxin-stimulated respiration is the result of an increased utilization of high-energy phosphate is supported by recent experiments in which the respiratory increase was prevented by an osmotic inhibition of growth (27, 274). However, Marrè and co-workers have some evidence that the primary action of auxin can increase the ATP level in the absence of any effect on energy-utilizing reactions (221, 222, 223). They also claim that auxin can activate growth and respiration by virtue of its effect on the ascorbic-glutathione redox system (220, 224). Physiological concentrations of auxin do not cause significant stimulations of the oxidative and phosphorylative activities of isolated plant mitochondria (280, 349). On the other hand, there are reports that the pentose cycle (161, 311) and acetate oxidation *in situ* (262) can be stimulated by auxin.

Neither gibberellin (176, 373) nor kinetin (117) stimulates the respiratory rate very markedly, although seeds soaked for 48 hr. in gibberellic acid show a higher respiration (269).

Work.—All the energy-requiring processes in plants are linked to the respiratory system. In some recent studies with respiratory inhibitors, this has been shown to be true for phloem transport (179, 376, 392), sugar absorption (139, 235), amino acid uptake (40), and the maintenance of a bioelectric field (208). The important problem of the relationship between respiration and salt accumulation will be covered elsewhere in this volume (193).

Phytopathology.—It is well known that fungus infections of plant tissues frequently cause a stimulation of the respiratory rate. During the black-rot infection of potatoes, there are also significant increases in the tissue organic phosphate and acid-insoluble nitrogen, the protein nitrogen of all the cell fractions, and the oxidative and phosphorylative activity of isolated mitochondria [Akazawa (1, 2); Akazawa & Uritani (7)]. These results suggest that an increase in the synthetic activity during infection is responsible for the changes in respiratory rate. This conclusion is supported by experiments on infected safflower [Daly & Sayre (72)]. The hypothesis that infection leads to an "uncoupling" of respiration is open to question (254, 362). There is some evidence that rust-infection of safflower leads to a greater contribution of the pentose cycle in carbohydrate breakdown (73). Infection may cause a change in response to respiratory inhibitors (95, 180, 363) or in the amounts of extractable oxidases (180, 181, 246, 296, 303), but the physiological significance of these effects is not clear. *Fusarium* culture filtrates (189) and toxins (277) have been tested for their effects on the oxygen uptake of intact tissues and isolated mitochondria, respectively, but the results were variable in both cases.

Among the wide variety of miscellaneous agents and treatments which

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have been used in recent studies to stimulate tissue respiration, at least temporarily, are the following: borate (273), HF (244), petroleum oils (147), and ozonated hexene (93, 359)—this last effect was also demonstrable with isolated mitochondria (103); a sudden increase in temperature (291) or storage at low temperature (312); geotropic stimulation (305); gamma-(123), X-(305), and UV-irradiation (276)—this last effect was reversible by visible light.

The Respiratory Apparatus

Information on the structure and composition of the respiratory apparatus, i.e., the mitochondria, is essential to an understanding of mechanisms, and several recent reviews of this subject have appeared [Dangeard (74); Hackett (126); Steffen (338)]. Since microscopic examinations of intact cells and isolated cell fractions have revealed several types of particles in the characteristic mitochondrial size range, there is some question as to the heterogeneity of this fraction. In living epidermal cells, only the mitochondria (spherical and rod-shaped) stain with tetrazolium and Janus green B [Sorokin & Sorokin (333)]. In some cells, e.g., elongating pea stems, all of the mitochondria are spherical in shape [Sorokin (332)], while in others, this is the predominant form (98, 243). Perner (278) has recently summarized the morphological evidence for the presence of another distinct type of particle, the "spherosome." These refractile, lipoprotein spheres show a characteristic submicroscopic structure, including an external membrane and dense inner core, by which they can be distinguished from the mitochondria and the structureless fat droplets [Strugger (344, 345)]. Recent studies with yeast and other fungi have likewise shown the presence of distinct "spherosomes," both in intact cells (23, 24) and in the isolated mitochondrial fractions (25). The exact biochemical role of these particles and their relation to the mitochondria remain to be established. Bautz (26) has concluded that the "spherosomes" are not respiratory centers, and this is supported by the recent demonstration that cytochrome oxidase is not associated with the refractile granules in yeast (144). It would be of interest to know whether they bear any relationship to the "lysozomes" of animal cells, which they resemble in size and in behavior during cell fractionation (78).

Observations on the ultrastructure of the mitochondria have recently been extended to a variety of higher plant tissues, including the roots of beet (97), onion (344), bean (146), wheat (149), corn (146, 209), pumpkin and barley (283); beet petioles (149); peanut cotyledons (183); barley embryos (369); skunk cabbage spadix (130); bean leaf (168), and *Gingko* prothallus (57). Although external and internal membranes are present in all cases, the internal configuration has varied a great deal. Thus, in higher plants (130, 149, 283) and in algae (62, 216, 369) the internal membranes may appear sinuous or tubular, suggesting the presence of microvilli or tubules, rather than the system of parallel "cristae" found in many mammalian cells (325). This point is particularly well illustrated in Heitz's (145, 146) electron micrographs, and he suggests that the tubules or bladders arise as a result of an

inward folding of the inner mitochondrial membrane. There are reports that the mitochondria can differentiate during development, changing from a homogeneous internal structure to one with "cristae" or "septa," which may subsequently become less dense and vesiculated [Von Wettstein (369); Lund, Vatter & Hanson (209)]. Where the mitochondrial ultrastructure has been examined both in tissues and in isolated fractions, a satisfactory correlation has been established; nevertheless, it is clear that the isolation procedure causes some structural modifications (97, 149, 183, 209).

The osmotic behavior of mitochondria has been demonstrated by determining changes in weight on exposure to solutions of different tonicities (3, 352). More data is now available on the chemical composition (lipide, protein, nucleic acid) of isolated mitochondria and microsomes [Martin & Morton (231, 232); Kmetec & Newcomb (183); Akazawa & Beevers (3)].

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PHOTOPERIODIC CONTROL OF FLORAL INDUCTION[1,2,3]

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INTRODUCTION

The physiology of flowering has been treated twice in this series: first, in 1952 in a very comprehensive manner by Lang (104) and secondly, in 1955, with an emphasis on *Xanthium* and consequently on American literature by Liverman (123). The present review is wider in scope than Liverman's and therefore in many cases goes back to the review of Lang, but it is not as comprehensive as the latter because the subject of vernalization, which is treated in this same volume by Chouard, has been omitted. The authors are not aware of any other comprehensive treatises on the subject written during the last six years, apart from the yearly reviews of the literature by Lang in *Fortschritte der Botanik* (105, 106, 107, 108, 113) which have been very useful in the preparation of the present review. Notable literature discussions are also found in some research papers (e.g., 98, 176, 221).

For obvious reasons most of the work on flowering has been done with plants in which this process is under strict control by the environment. Of the papers under review, most deal with short-day plants (SDP), a much smaller number with long-day plants (LDP), while little work has been done with plants in which flowering is not affected by daylength. Consequently, much more is known about floral induction in SDP than in LDP, while insight into flowering of "day-neutral" plants is very limited indeed. In SDP, formation of a mobile floral stimulus in the the leaf during the dark period is no doubt the crucial process in induction, although the nature of the stimulus is unknown. There is evidence that a similar or even identical stimulus is also formed in LDP, but whether this is also produced during a specific part of the inductive cycle is uncertain. Finally, in day-neutral plants a transmissible floral stimulus has been shown to occur in some species, but in others efforts to detect it have so far met with failure. For the purpose of the present review floral induction has therefore to be defined rather loosely as the chain of processes preceding and leading to flower initiation (the visible shift from vegetative to generative activity of the apical or axillary meristem). It has often been suggested that the physiological mechanism involved in floral induction is essentially the same in all higher plants and that the differences in response

[1] The survey of literature pertaining to this review was concluded in August, 1958.

[2] The following abbreviations will be used: CL (continuous light); 2,4-D (2,4-dichlorophenoxyacetic acid); Ga (an unspecified mixture of gibberellins); GA_3 (gibberellic acid); IAA (indole-3-acetic acid); LDP [long-day plant(s)]; NAA (α-naphthaleneacetic acid); SDP [short-day plant(s)].

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to external stimuli, although often striking, are not of crucial importance. For the moment, however, too little is known about the physiological backgrounds of flowering in a wide range of species to permit acceptance of this theory, and to devise a scheme into which the evidence concerning plants of different types of response could be fitted. For this reason, SDP, LDP and day-neutral plants will be treated separately. For the rest, the organisation of the review will be left to speak for itself. Factors influencing flower differentiation and subsequent stages of flowering will not be discussed. One of the main reasons for this limitation is that much of this subject matter has been covered in a recent review on sex-expression by Heslop-Harrison (71).

JUVENILE PHASE

In *Arachis hypogaea* flower primordia are formed in the axils of the cotyledons, and can be detected shortly after germination (47, 156). Such cases appear to be rare, however. Usually, the seedling has to reach a certain size before flowers can be formed. This size can be quantitatively expressed as the minimum number of leaves laid down before flower initiation. Various hypotheses have been proposed to account for this "juvenile phase." Zieriacks (224) reasoned that a certain minimal leaf area is required for the production of a sufficient amount of floral stimulus, and concluded that the minimal leaf number is the sum of the number of leaves required to reach this critical area and the number of leaf primordia formed until the moment the stimulus reaches the meristem. She showed that in several SDP and LDP the cotyledons suffice for flower initiation in the inductive daylength. In *Kalanchoë blossfeldiana*, however, some leaves have to be present as well, as the critical leaf area of 100 mm.^{2} is much greater than the area of the cotyledons.

This simple hypothesis cannot account for all cases, however. The following complications may arise: (*a*) Cotyledons and primary leaves may show a reduced sensitivity to inductive conditions. At an age of three months, plants of *Scabiosa ukranica* need three weeks of continuous light to flower, while nine-month-old plants require only one week (36). In *Xanthium pensylvanicum* the cotyledons, which have an area of 9 cm.^{2}, do not perceive the short-day stimulus, although 7 or 8 cm.^{2} of foliage leaf suffice for flower induction in an adult vegetative plant (87). Zeevaart (221) showed by grafting experiments that in *Perilla crispa*, leaves formed on the second node need about twice as many short-day cycles to reach a state in which they can induce an adult plant to flower as leaves from the fifth node. (*b*) In a young plant, the ratio between mature and immature leaves is low. Immature leaves may not only be less sensitive to inductive conditions, but in some cases actually inhibit floral induction. An example of this is the soybean (44, 46). (*c*) The seed may contain flower promoting as well as flower inhibiting substances. This was shown to be the case in *Pisum sativum* (69) and will be discussed later in some detail. (*d*) One should not overlook the possibility that cases exist where the cotyledons and primary leaves produce the floral stimulus but this is not transported to the growing point, or does not elicit a

response in the latter. However, there is some evidence against this possibility. In the LDP *Sempervivum alpinum* (5) and *Frageria vesca semperflorens* (176) daughter rosettes flower at a much earlier date when attached to the parent plant than when separated from it. This shows that the floral stimulus is transported to and induces a reaction in meristems of the young plant long before its own leaves can produce this stimulus themselves.

Holdsworth (75) has discussed the older literature concerning the juvenile phase, in which the following concepts are found: the minimum leaf number is morphologically determined; it corresponds to a specific degree of anatomical maturity; it represents the leaf area required for a specific amount of carbohydrate (whether or not in relation to other substances, e.g., nitrogen); it is the outcome of the fact that generative growth lingers behind vegetative growth because flower induction takes time, or is inhibited by the cotyledons; the minimum leaf number simply represents the number of primordia present in the seed. Most of these concepts are rather vague and lack the support of experimental evidence.

SHORT-DAY PLANTS

The First High-Intensity Light Process

According to the generally accepted definition by Lang (104, p. 284), SDP are plants in which the daily dark periods induce or accelerate flower formation. However, under normal conditions the light preceding the dark period, as well as that following it, plays an active part in floral induction. In analogy to Liverman's review (123), the evidence concerning the processes occurring before, during, and after the dark period are treated under separate headings.

Light intensity.—When the light intensity during the photoperiod is lowered, flowering response elicited by the subsequent dark period decreases (170). Many SDP remain vegetative at very low light intensities or when days are extremely short. This inhibitory effect on flowering can be overcome by application of sugar. When sugar is supplied, some SDP, viz., *Chenopodium amaranticolor* (128) and *Pharbitis nil* (188) have been shown to flower even when raised in continuous darkness. From these data it has been concluded that the primary function of the photoperiod preceding an inductive dark period is the production of photosynthates (104, 123). However, not all SDP react in the same manner. De Zeeuw (213, 214) showed that when plants of *Perilla crispa* were subjected to low light intensity the critical length of the dark period was shortened until at an intensity of 300 μw./cm.^{2} flowers were initiated even in continuous light. In one series of experiments two-branched plants were grown in 16 hr. photoperiods, which normally are not inductive, one branch being subjected to high light intensity, the other to low intensity. The latter not only flowered itself but also induced flowering of the branch kept at high light intensity.

Wavelength of light.—Three methods have been used to study the effect of light of limited spectral regions, namely (*a*) a photoperiod consisting of

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coloured light, (*b*) coloured light given as an interruption of a "white" photoperiod and (*c*) coloured light given immediately after a white photoperiod.

(*a*) Meijer (134) subjected the SDP *Salvia occidentalis* to long or short periods of light of different spectral regions. In cycles of 10 hr. of light and 14 hr. of darkness the plants flowered regardless of the colour of the light during the photoperiod. In cycles of 16 hr. of light and 8 hr. of darkness the plants remained vegetative in white, blue, or green plus infrared radiation but flowered in green and red light. The effect of red light was dependent on intensity; plants subjected to 360 μw./cm.^{2} flowered, but those which received 1200 μw./cm.^{2} remained vegetative. However, this may have been due to the fact that the red still contained about 2 per cent infrared. Plants of the same species were also subjected to 10 hr. of light and a 14 hr. dark period interrupted in the middle by a 10 min. light break (135). This light break annulled the effect of the night (i.e., the plants remained vegetative) but only when the main photoperiod consisted either of white or blue light (infrared was not investigated in this series of experiments). As reliable equipment for research on this aspect has been developed only recently, these experimental data are probably just the first of more comprehensive series. It will be of special interest to see how the different wavelengths affect the critical night length.

(*b*) Könitz (18, 99) subjected *Chenopodium amaranticolor* to a daily light period of 13 hr. of white light interrupted at various moments by different quantities of infrared radiation (7050–9800 A). Flowering could be suppressed by $3\frac{1}{2}$ hr. infrared radiation (200,000 kiloergs/cm.^{2}) given 5 hr after the beginning of the photoperiod. When the interruption was given at an earlier or later time, a higher amount of energy was required. Red light given immediately after the infrared radiation could completely reverse the inhibitive effect of the latter. The amount of red light energy required for this reversal was relatively small (29,800 kiloergs/cm.^{2}), and did not vary with the amount of infrared required to produce the original inhibitory effect. A similar reversible photoreaction was found some years ago for the light interrupting a dark period, as will be discussed below. It is not known yet whether the pigment systems involved are identical.

(*c*) In another experiment of Könitz (99), plants of *C. amaranticolor* were subjected for 12 hr. of white light followed by 3 to 10 hr. of light of various spectral regions. It was found that the critical night length was 8 hr. with white, 9 hr. with red, and only 4 hr. with blue supplementary light. Infrared had no effect; even plants in 12 hr. of white plus 10 hr. of infrared (the maximum duration given) flowered at the same rate as the controls. In older experiments by Borthwick *et al.* (8) it had been found that 30 min. of infrared radiation given immediately after the white photoperiod shortened the critical night length of *Xanthium pensylvanicum* by about 2 hr. An attempt to obtain similar results with *Chrysanthemum morifolium* failed (34). This aspect is now also being studied by Meijer and others and a discussion can better be postponed to a later date when their results have been published.

Relative length of photo- and nyctoperiod.—It has been known for a number

of years that the response of SDP to an effective dark period depends to a certain extent on the preceding light period; when the latter is extended, flower induction by a given dark period decreases. Conversely, when the dark period following an optimal photoperiod is extended, its effectiveness also falls off (104). In 1953, Wareing (198) showed that there is a relation between the length of the optimal light period and the duration of the dark period. In his experiments with Biloxi soybean the optimal light period, which was 16 hr. when the dark period lasted 12 hr., rapidly fell off to 10 hr.when the latter was extended to 27 hr. Blaney & Hamner (6) confirmed the general trend of these results and showed that the converse is also true: with a given effective photoperiod there are lower-critical, optimal, and upper-critical night lengths, and these become longer when the accompanying photoperiod is shorter.

To account for this inverse relationship of the critical and optimal values of light and dark period, Blaney & Hamner suggested that light and dark interfere with a system that is subjected to a diurnal rhythm. When plotting the number of nodes bearing flower buds against cycle length, they found that optimal flowering occurred in cycles of a duration of either 24 hr. or 48 to 52 hr. long, independently (within certain limits) of the relative duration of light and darkness during the cycle. They suggested that a process occurs in the plant that passes rhythmically through phases of increasing and decreasing ability to inhibit or inactivate the flowering stimulus. These results agree with Bünning's theory of endogenous rhythms, which will be discussed later.

Interactions of long and short day.—In 1954, Harder & Bünsow (60) found that the length of the photoperiods at which plants of *Kalanchoë blossfeldiana* were grown before the inductive treatment affected the flowering response. The number of flowers formed as a result of a given short-day treatment was found to decrease with an increasing length of the preceding long days. Schwabe (172, 173) showed that twelve short days, which when given in a row cause a high degree of floral induction, have no effect whatsoever when given in alternation with long-day cycles. This shows that during long-day cycles not only no floral stimulus is produced, but that also a process takes place that annuls the flower-promoting effect of one or more short-day cycles. In a more detailed study of this process it was found that each intercalated long day, quite apart from being noninductive itself, led to the destruction of the effect of 1.5 to 2 short days. A similar figure was found with Biloxi soybean and *Perilla crispa*. The effect of the long days could be counteracted either by lowering the temperature prevailing during both the light and the dark portion of the cycle, or by following the long day immediately by a dark period of 24 hr. A 24 hr. dark period preceding the long day hardly increased flowering, which proves that the long day exercises its inhibitory effect on the subsequent and not on the preceding short-day cycles, and that this inhibition is annulled by a following long dark period. Two other facts are worthy of note. In the first place, the inhibitory effect was also obtained when, instead of a long photoperiod, a short day was given with a

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short light-break in the middle of the accompanying dark period. Secondly, the amount of inhibition was proportional to the amount of induction given. Each extra short day given almost doubled the flower number, while each intercalated long day reduced by more than half the number of flowers to be expected from a given number of short-day cycles.

Carr (31) found that in *Chenopodium amaranticolor* long days given in alternation with short days did not annul the inductive effect of the latter but caused a considerable delay in flowering, which was still greater when single short days were intercalated with two or four long days. In Biloxi soybean alternation of single short days and single long days prevented induction. When pairs of short days were alternated with long days the number of flowers was reduced in comparison to the controls, but this reduction was about the same whether one, two, or twelve long days were intercalated. Wellensiek (206) interrupted a short-day treatment at various times by 48 hr. of continuous light (CL) and measured the time required for the appearance of flower primordia in *Perilla crispa*. A new fact brought out by this work is that the inhibitory effect of the CL depends on the moment of the inductive period at which it is intercalated. A maximum delay of six days was obtained when the CL was given four to eight days after the beginning of the short-day treatment, that is at about the beginning of flower initiation. From then on its effect gradually decreased until 48 hr. of CL, when given after about twelve short days, delayed flowering only by two days; in other words, CL no longer had either a positive or a negative effect on induction. The inhibitory effect of CL did not occur when the temperature was simultaneously lowered to 5°C.

The Dark Process

Temperature.—One of the arguments for the assumption that in SDP the crucial process leading to the production of the floral stimulus occurs during the dark period is that flowering is inhibited to a much greater extent by low night temperatures than by low temperatures during the day (104). This has been confirmed by Wellensiek (202) who showed that flowering of *Perilla* was completely suppressed when the night temperature was 5°C. and the day temperature 20°C., but only slightly retarded when these temperatures were reversed. In *Kalanchoë blossfeldiana* no induction by short days takes place at 10°C. (157). However, the rule that low temperature decreases the effect of inductive cycles apparently does not apply to all species. The strawberry *Fragaria virginiana*×*chiloense* "Marshall" is an SDP at high temperature, but at 6–10°C. flowers in any daylength (208). Similarly, *Euphorbia splendens* flowers in 10 hr.-days but not in 15 hr.-days when the temperature is 20°C. or higher, but it flowers in both daylengths at 15°C. or below (158). This can be explained by the assumption that floral induction is determined by two processes, both with a $Q_{10} > 1$, viz., a promotive process occurring during the dark and an inhibitory one occurring during the light. The effect of low temperature then depends on which process is inhibited most. In *K. blossfeldiana* the

optimum and minimum temperatures for the inductive short-day process are 20° and 10°C., respectively, but the inhibitive long-day process has an optimum above 25° and a minimum below 10°C. (157). In the strawberry, on the other hand, the thermal minimum of the promotive process apparently lies below the minimum of the inhibitory one. When plants are subjected to 8 hr. of daylight supplemented by 16 hr. of artificial light, there is an inverse relationship between temperature and light intensity required to suppress flower initiation: the lower the temperature, the higher the light energy required to prevent flowering (208).

The inductive reactions during the long dark period can also be inhibited by high temperature. *K. blossfeldiana* remains vegetative at 30°C. (157). Schwemmle (174) found that 35°C. given during the first or second 3 hr. period of a 12 hr. night promoted induction, but given during the last 3 hr. of the dark period completely inhibited flowering. These results were interpreted in relation to endogenous rhythms and will be referred to again later. Nakayama (140) gave a relatively short period of 34–36°C. at various times during a 16 hr. dark period and observed that the inhibitiory effect on flowering of *Pharbitis nil* reached a maximum when the high temperature was given from the twelfth to the fourteenth hr. of the night. Before and after, the effect was much weaker.

Effect of light interruption.—One of the most effective ways of studying the processes occurring during the dark period has been interruption of the night by light breaks of various spectral composition and given at different times. There is much literature on the subject (104, 123) and only the most recent papers will be reviewed. In studies with *Xanthium pensylvanicum*, Salisbury & Bonner (165, 166) found that the maximum effect of a light interruption was obtained when it was given 8 hr. after the beginning of a dark period, regardless of the length of the latter. This was explained by the assumption that the synthesis of the floral stimulus, which is light stable, does not begin until after 8 hr. of darkness, while the preparatory stages are completely reversed by the light, so that after a light break the whole process has to start afresh. This assumption also explains why light breaks are less effective when given closer to the beginning or to the end of a 16 hr. dark period. In the first case it is the length of the remaining dark period, in the second that of the preceding dark period which determines the amount of floral stimulus produced. In some experiments dark periods longer than 13 hr. were less effective in promoting flowering than shorter ones. In these cases a light interruption given in the early part of the dark period actually promoted flowering, which can be explained by assuming that the light break annulled the effect of the preceding darkness and thus shortened the excessively long dark period. Although the effect of a light interruption is strongly dependent of the time of the night at which it is given, the amount of light required to elicit the maximal response obtainable at a given time was shown to be constant, viz., about 108 kiloergs/cm.^{2} of red light. This was interpreted as an indication that the time the first light interruption was given, viz., 3.25

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hr. after the beginning of darkness, the conversion of the infrared absorbing pigment to the red-receptive form (see below) was completed. On the other hand, Könitz (18, 99) found with *Chenopodium amaranticolor* that complete suppression of flowering induced by a 14 hr. dark period was possible by interruptions of red light given between 3 and 10.5 hr. after the onset of darkness, provided the amount of light supplied was high enough. Just before the middle of the night 240 kiloergs/cm.^{2} of red light could prevent induction, but this amount increased towards the beginning and the end of night until 3 hr. after the beginning, or 2.5 hr. before the end, 86,400 kiloergs/cm.^{2} were required.

Red light was used in these experiments because it has been known for a number of years that the action spectrum of the radiation that annuls the effect of darkness shows a pronounced maximum at 6500 A. Infrared radiation near 7350 A reverses the action of the red light, provided the interval between red and infrared radiation is not too long. The pigment system involved plays a role not only in flowering but also in many other physiological processes that are affected by light. A discussion of all that is known about the system would necessarily include the work on these other processes also and will therefore not be attempted. The reader is referred to the existing reviews [e.g. (70, 123, 201)].

SDP tested for the reversible photoreaction are *Xanthium pensylvanicum* (8), *Glycine max* var. "Biloxi" (42), *Amaranthus caudatus* (42), *Chrysanthemum morifolium* (34), and *Chenopodium amaranticolor* (99). Downs (42) showed that in *Xanthium* and Biloxi soybean the degree of repromotion by infrared irradiation decreased when the interval between red and infrared was increased, until after 30 to 45 min. repromotion was no longer possible. When red-infrared cycles were given in rapid succession, the degree of repromotion also decreased with each cycle until after about four cycles no repromotion could be attained. The data obtained with *Chrysanthemum* (34) show the same trend. A possible explanation is that a relatively short period is sufficient for the red-absorbing form of the pigment to build up a condition inhibitory to flowering. This process is dependent on the temperature, for at 5°C. the loss of the possibility of repromotion of flowering was much slower than at 20°C. Short periods of infrared given during the dark period have no effect on flowering but greater amounts of infrared have an inhibitory effect and also fail to repromote flowering of plants that received an inhibitory treatment with red (34).

Role of dark fixation of CO_2.—Some years ago it was shown that the pattern of CO_2 fixation by *Kalanchoë blossfeldiana* is regulated by the photoperiod (54, 191). After about ten short days the CO_2 uptake in the dark increases markedly while there is a rapid evolution of CO_2 at the onset of the light period. After 33 short days there is a net uptake of CO_2 during the dark periods and a net release during the day, which constitutes a reversal of the normal pattern of CO_2 exchange. If the dark period is interrupted in the middle by a short period of full light, this increase in dark fixation is pre-

vented. High temperature (30°C.) during a long dark period leads to evolution of CO_2. As both light interruption of the night and high night temperature inhibit floral induction, there are close parallels between this process and CO_2 fixation. Whether these parallels are the outcome of a causal relationship has since been the subject of further study.

Kunitake *et al.* (101) showed that $C^{14}O_2$ fixed by induced leaves is incorporated in the same compounds as in leaves from plants grown in long days, and that the relative distribution of radioactivity in these compounds does not show any significant differences either. This shows that short-day treatment neither creates a new pathway for CO_2 fixation nor changes the relative rate of CO_2 transfer between the different compounds. It was suggested that if increased dark fixation of CO_2 under short-day conditions is causally related to floral initiation, the relation is a rather nonspecific one.

Dark fixation has also been studied in Biloxi soybean and *Xanthium pensylvanicum* (115, 175). In these plants the pattern of labeling is quite different from that in *Kalanchoë*. Another contrast to this plant is that differences were found in the relative activities incorporated into the different compounds between vegetative and induced plants. These differences existed both in the products of dark fixation of $C^{14}O_2$ and in those of steady-state photosynthesis. However, none of the compounds concerned is known to have any particular significance in floral induction. As in *Kalanchoë*, the rate of CO_2 fixation decreased in the course of the dark period. In the light it decreased with increasing length of the preceding dark period.

The question of whether a causal relationship exists between the pattern of CO_2 fixation caused by short-day treatment and the short-day-induced flowering response must for the moment remain unanswered. Exclusion of CO_2 from the atmosphere during the dark period results in a reduction of the number of flowers in soybean and *Xanthium* (115) but this may be due to an effect on flower differentiation or development and does not necessarily mean that induction is affected. The most spectacular increase in dark fixation in *Kalanchoë* occurs at a moment when floral induction has already taken place. One can agree with the conclusions of Sen & Leopold (175) "that there are compounded interactions between the light and dark periods in photoperiodism, and that these interactions are reflected in altered patterns of CO_2 fixation both in light and darkness," but the problem of whether and how these patterns specifically affect floral induction awaits further study.

The induced state.—Zeevaart (217) grafted leaves of *Perilla crispa*, which had received an inductive short-day treatment, on vegetative plants under long-day conditions. He found that the leaf not only induced flowering of this vegetative stock but also of others on which it was subsequently grafted. As a matter of fact, the leaf retained its full capacity to induce a vegetative plant until, after having been grafted and regrafted on seven stocks in the course of three months, it became senescent and died. From this it was concluded that after a sufficient number of short-day cycles the leaf is in an induced state (i.e., it has the ability to produce the floral stimulus) which is not lost in any

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subsequent light regime. In addition it was shown that the induced state [previously called "photoperiodic impression" by Lona (127)] is not lost either by soaking in $10^{-5}M$ solution of the auxin naphthaleneacetic acid (NAA), dinitrophenol or sodium azide, nor by a heat treatment at 45°C. for 5 hr. It will be difficult to establish whether the same holds true for other SDP, because the leaves as a rule do not stand grafting and regrafting so well as those of *Perilla*.

Leaves of *Perilla* can be brought into the induced state in the absence of buds (7) or even when detached from the plant (218, 221). It has been claimed (29, 30) that this is not possible in *Xanthium*, which is probably attributable to the fact that it is very difficult to keep detached leaves of this plant in a good condition for a long enough period. In *Xanthium*, rapidly expanding leaves are the most sensitive to the inductive treatment (93), and detached leaves show hardly any expansion and soon become chlorotic. When only those leaves are selected that have stood the treatment best, these will induce flowering in vegetative stocks (221). However, a detached *Xanthium* leaf will not act as a donor when it has been subjected to only one long dark period, although this is sufficient when it is left on a plant with active buds. This is probably because the floral stimulus is broken down in the leaf in *Xanthium* (126) so that during the time required for the establishment of a graft union a marginal amount of stimulus has decreased below the critical value. In the light of this and other evidence it does not seem very probable that the induced state is as indestructible in *Xanthium* as it is in *Perilla*. Another and more striking difference between the two plants is that in *Xanthium* actively growing buds or very young leaves can be induced to produce the floral stimulus themselves by a leaf subjected to short-day treatment, even in long days (123). In *Perilla* the induced state is strictly localized in the leaf or part of the leaf that has been subjected to the inductive environment (221).

The Second High-Intensity Light Process

When plants of *Xanthium* after an inductive dark period of 12 hr. are subjected to a light flash followed by a second dark period of 5 hr., flowering is reduced in comparison to that of plants moved directly to high-intensity light. This depresssion of flowering by the second dark period is absent when, instead of a light flash, 5 hr. of high-intensity light are given. No flowering occurs when the leaves are cut off after 12 hr. of darkness plus 5 hr. of light, which shows that after this treatment the stimulus is still in the leaves. This evidence led Lockhart & Hamner (125) to the conclusion that a period of high-intensity light is required to stabilize a precursor of the floral stimulus which is broken down in the leaf in the absence of light (although only to a certain extent, as *Xanthium* flowers eventually in continuous darkness). The inhibitory effect of the second period of darkness did not occur at 4–6°C., but the promotive effect of high-intensity light was not decreased by low temperature. With regard to the nature of this light process Liverman (123) sug-

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gested that it might be (*a*) a variant of the low-intensity light reaction, in which case the inhibition should be reversed when immediately followed by exposure to infrared, (*b*) comparable to the first high-intensity light process, in other words, consist primarily of photosynthesis, or (*c*) an independent light process whose nature is at the moment unknown. Meanwhile, Carr (32) has collected evidence in favor of the second of these possibilities. His results confirm that the flowering response is proportional to the intensity of the light given immediately after the dark period. This light primarily affects the leaf, but darkening the terminal bud also reduces flowering. This suggests that the light may not be essential for the conversion of a precursor in the leaf, but rather may act on the transmission of the floral stimulus from the leaf to the bud. This was confirmed when it was found that a supply of sucrose to the leaf completely obviates the need for high-intensity light following the dark period. Carr suggests that the floral stimulus moves in mass flow with the stream of assimilates, which may be supplied by photosynthesis or by immersion of the leaf in sugar solution. This hypothesis is supported by work on the translocation of 2,4-dichlorophenoxyacetic acid (2,4-D) which is reviewed in some detail in Carr's paper. No data are available on the second high-intensity light process in other plants than *X. pensylvanicum.*

Translocation

The translocation of an active principle from the leaves to the buds, either through an intact stem or across a graft union, is the principal proof for the existence of a floral stimulus. It is known that this stimulus moves mainly in the phloem with the stream of assimilates. Especially when transmission has to take place against the usual basipetal stream of assimilates, flower initiation is almost completely inhibited by the presence of noninduced leaves on the receptor part of the plant, and only occurs when a food deficit is created by the removal or darkening of these leaves. Growing shoots on the donor part must also be removed, probably because otherwise the main stream of assimilates will be directed towards these consuming sites and insufficient amounts of floral stimulus will reach the receptor (104, 221). Although the inhibitory effect of noninduced leaves on transmission of the floral stimulus can usually be explained in terms of an effect on the mass flow of assimilates, the possibility that they exercise another, more specific effect cannot be excluded (121). In strawberry plants the stimulus moves from an induced mother plant through the stolon to a daugher rosette kept in long days (64), but it has been shown that the latter forms an active principle that is also transmitted through the stolon and depresses flowering of a rosette kept in short days (57).

In *X. pensylvanicum* the product of the dark reaction normally moves out of the leaf during the day following the inductive dark period. When the induced leaf is cut off immediately after the dark no flowering occurs. A few hours of daylight are required for the stimulus to move out of the leaf in effective amounts. The transmission-rate is proportional to the light in

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tensity. Under the usual experimental conditions transmission appears to be completed after about 12 to 16 hr. (123, 177). Similar results have been obtained with *Pharbitis nil*, another SDP that requires only one long night to flower and consequently lends itself to this type of experiment (79, 81, 83, 84). With the latter plant Imamura *et al.* (79, 80) have devised an intricate but elegant method to determine the transmission-rate of the floral stimulus, which was found to be 2.6 to 3.8 mm. per hour. A graft union apparently does not slow down transport (82). It is of course impossible to determine the moment at which the first floral stimulus reaches the bud; one measures the time at which a sufficient amount has arrived to elicit a response. This time is increased, because some stimulus is lost during transport (86). This may explain why the figures found are low in relation to the known transmission-rate of carbohydrates. That the floral stimulus is indeed transported by the mass flow of assimilates was confirmed by Zeevaart in graft experiments with *Perilla crispa* (221). He found that flowering of the receptor plants could not be obtained when the duration of the contact of donor and receptor had been shorter than necessary for the transmission of C^{14}-labeled sucrose.

Effect of Growth Regulators

Auxin.—At the time the previous reviews were written, the available evidence warranted the assumptions that auxin inhibited flower initiation of SDP and that it exercised its effect by acting on the synthesis of the floral stimulus. Since then new light has been thrown on the subject and the prevailing opinions have been modified. The evidence concerns (*a*) the amounts of native auxin in the plant in different photoperiods and (*b*) the effect of application of synthetic auxin.

(*a*) The older evidence suggests that the auxin level in the leaf increases during the light period and decreases during the night, so that the plant contains more auxin in long-day than in short-day cycles. However, Vlitos & Meudt (194) using quantitative paper chromatography found that plants of Biloxi soybean contained considerably more indole-3-acetic acid (IAA) when grown in 8 hr.-days than in 18 hr.-days. In Lincoln soybeans, which have only a quantitative short-day requirement, the difference was slight. Both plants had a much higher content of indolepyruvic acid (a precursor of IAA) in short days than in long days. Von Guttenberg & Zetschke (56) studied the auxin content of *Helianthus annuus*, *Lycopersicum esculentum* and *Nicotiana tabacum* in the course of a dark period. They found a steady increase, leading to considerable amounts of auxin in long dark periods. In *Brassica oleracea*, which contains no IAA but a neutral growth substance, auxin content was high in the light and did not increase in the course of a dark period. Phillips & Wareing (148) studied the auxin content of the leaves of trees in short and long day and found no differences in *Robinia pseudoacacia*, *Betula pubescens* and *Acer pseudoplatanus*. However, the last species contained more growth inhibitor in short days.

When this evidence is taken in its entirety, the only conclusion that can

be drawn is that the effect of light and darkness on auxin content depends in the first place on the species involved. Different plants contain different auxins and inhibitors which may not all be affected by the environment in the same way. It is conceivable that conflicting data can be brought into agreement when the specific chemical nature of the substances involved is determined, which is now possible by paper chromatography. However, this will not be easy, as demonstrated by the fact that Vlitos *et al.* (196, 197) could not find IAA in *N. tabacum* by this method but that this substance was nevertheless shown to occur in this plant by Kefford & Helms (91). Further knotty problems are: the importance of bound versus free auxin, the effect of the environment on auxin transport and the possibility suggested by Becker (4) that the fluctuations in auxin content (of *Kalanchoë blossfeldiana*) are not caused by the alternation of light and darkness but by an endogenous diurnal rhythm.

(*b*) The effect of applied auxin on flowering has recently been reviewed by Leopold (117). The evidence pertaining to SDP is briefly as follows: Auxin (NAA) applied in weak concentrations (0.001 to 1.0 p.p.m.) to the seed promoted subsequent flowering under inductive conditions of *Euchlaena mexicana* and *Glycine max* var. "Biloxi," provided the auxin treatment was followed by low temperature (3°C.) during two weeks. The same substance, applied in concentration of 5 p.p.m. in lanoline paste just before induction, had a significant promotive effect on flowering of Biloxi soybean and *Xanthium canadense*. Flowering of Biloxi soybean could also be promoted when weak concentrations of NAA were applied during the inductive period, but only when the the temperature was simultaneously lowered to 10°C. (216). In the course of a study on the effect of auxin applied during the inductive dark period to *Xanthium pensylvanicum*, Salisbury (164) found a hardly significant promotive effect on flowering of weak concentrations of IAA and NAA. In conformity with much of the older evidence (123) it was found that larger quantities inhibited induction in a degree proportional to concentration. An interesting new fact that was brought out is that the auxins did not affect the critical nightlength. The inhibitive effect reached a maximum when NAA was applied 2 to 3 hr. after the onset of darkness. From then on its effectiveness gradually decreased until it became ineffective when applied some hours after the plants had been returned to the light. It could be shown by defoliation that this time coincided with the stage at which the total amount of the stimulus had left the leaf. It was concluded from this evidence that auxin affects primarily the breakdown of the floral stimulus in the leaf, although the possibility is left open that it also inhibits the transmission of the stimulus (163 to 166).

Gibberellin.—Since gibberellin (Ga) was shown to induce flowering under adverse conditions in a number of cold- or long-day-requiring plants (112), this group of substances has been applied to a number of SDP also. So far, however, no cases have been reported where Ga could induce flowering of SDP in long day. In a variety of *Chrysanthemum morifolium* requiring both

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vernalization and short day to flower, Ga substituted for low temperature but not for short days (41). In *Kalanchoë blossfeldiana* Ga not only did not induce flowering in long days (61) but actually inhibited flowering of two-leaved plants in which one leaf was kept in the inductive daylength (62, 63). *Xanthium pensylvanicum* (55, 112), Biloxi soybean and *Perilla crispa* were not induced to flower in long day. The flowering response of *P. crispa* in short days was slightly depressed (129, 130). In *X. pensylvanicum*, Ga affected neither the flowering of intact plants nor that of disbudded plants with only mature leaves. The flowering of disbudded plants with young leaves, and of slow growing cotyledonary buds showed a threefold increase after application of gibberellic acid (Ga_3). It was suggested that Ga_3 promotes the capacity for the storage of the floral stimulus in the immature tissues (120).

Other growth regulators.—Maleic hydrazide, 2,2-dichloropropionic acid and 2,4-D inhibit flowering of *Xanthium* but only at concentrations which also cause an inhibition of vegetative growth or other toxic responses. The effect on flowering of these substances is also independent of the time of application (during or after the inductive dark period). From this it was concluded that they exercise an effect on the differentiation of the floral bud. The effect of 2,4-dinitrophenol was more specific, because it could be shown that this substance inhibits flowering only when applied during the dark period, from which it may be concluded that it has an effect on the synthesis of the floral stimulus. In contrast to the substances mentioned, cobaltous ions interfere with the mechanism which controls the critical nightlength (164).

Discussion

When the evidence concerning SDP is taken in its entirety, there seems to be little doubt that the physiological mechanism leading to floral induction is very similar in the various species belonging to this group. Nevertheless, there are some striking differences, the most important of which concerns the fate of the floral stimulus after its production in the leaf. In *X. pensylvanicum* the stimulus apparently is destroyed in the leaf (at least partly) when not transported in time to an active bud where it is preserved and perpetuated. In *P. crispa* on the other hand, the potency of the leaf to produce the floral stimulus, once induced, is stable; the active bud reacts to the stimulus by flower initiation but is unable to store or perpetuate it. As it has been reported (85) that in *Pharbitis nil* a bud is required for successful induction, this plant apparently reacts in a way similar to *Xanthium*. One might hypothesize that this is why these two plants need only one inductive cycle, i.e., only a small amount of stimulus from the leaf is required to "inoculate" the bud, while a steady stream of stimulus from the leaf is required in *Perilla* and presumably in many other SDP. The existence of these and similar differences among the various plants should be kept in mind whenever one tries to draw general conclusions from evidence concerning one or at most a few species.

The recent evidence concerning the action of the first light process does

not contradict the old hypothesis that this light promotes flowering, probably simply by the production of building material and respiratory substrate, but it has emphasized that this light has also an inhibitory effect on subsequent induction. In this connection the following observations are of particular interest: intercalated long days destroy the effect of one or more subsequent short-day cycles [in several species (172), (206)], critical nightlength is reduced when the light intensity is reduced during the preceding light period [in *Perilla* (214)], when this light period consists of red or green radiation [e.g., *Salvia occidentalis* (134)], or when during part of the day the temperature is lowered [e.g., *Xanthium* (215)]. From these data the conclusion may be drawn that the function of the dark period consists, at least in part, of the removal of an inhibitory effect built up during the preceding light period(s).

Concerning the nature of this inhibition two suggestions have already been made in the previous literature review (123), viz., (*a*) at the end of the photoperiod the red-infrared-absorbing pigment system is in the infrared-absorbing form, and (*b*) a long light period leads to a high concentration of auxin. The function of the dark period is then, first, to convert the pigment system to the red-absorbing form (166) and, second, to reduce the auxin concentration to a level which is promotive, or at least not inhibitory, to flowering. In the light of recent evidence the second possibility must be discarded because the auxin level apparently does not always increase in the light and decrease in darkness (56, 194), and auxin does not seem to affect the synthesis of the floral stimulus anyhow (164). The conversion of the pigment system still appears to be a prerequisite, because there is no doubt that it is of crucial importance in the inductive process. The available evidence about this pigment system gives an explanation of the effect of red or infrared radiation given immediately after the daily (white) photoperiod: infrared reduces the critical night length by converting the pigment system to the red-absorbing form, while red supplementary light has the opposite effect by keeping it in the infrared-absorbing form which prevents the inductive process. However, the fact that infrared or red radiation, when given during the whole length of the photoperiod, has the opposite effect (viz., an extension or a reduction of the critical nightlength, respectively) cannot be explained in these terms. Nor can the effect that infrared inhibits flowering when given in high energies during either the light or the dark period (34, 99). For a tentative explanation of this discrepancy one may choose between two possibilities. First, one may assume that one pigment system is involved, but that the plant passes rhythmically through phases of differential sensitivity, so that infrared inhibits flowering at a given time but promotes it when given 12 hr. later, while red has the opposite effect (99). The other possibility is that a second pigment system is involved. Evidence favouring the latter possibility is that when the photoperiod consists of light of a limited spectral region, green has an effect similar to red, and blue an effect similar to infrared radiation (134), which is not in accordance with the absorption spectrum of the known pigment system.

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The next step in the process from light perception to the production of the floral stimulus is completely unknown. As the red-infrared-absorbing pigment system is involved in a great many physiological reactions, it is improbable that it would act directly upon a system that is specific for flowering. It was one of the attractive properites of the theory proposed by Liverman (123) that it linked up the action of this pigment system with auxin metabolism, which is also catholic in its effects. However, since the role of auxin in the production of the floral stimulus has become problematical, this theory now hangs in the air.

The floral stimulus, whatever its nature may be, moves in mass flow with the photosynthates to the meristems. Whether or not it elicits a visible response depends not only on the quantity of stimulus but also on the condition of the meristem. If all growing points were equally sensitive to the stimulus, vegetative growth would forcibly come to an end once the plant is under inductive conditions. This happens in some species, but the fact that many others continue to grow shows that mechanisms exist by which buds may "escape" the floral stimulus. In some cases, e.g., in *Xanthium* (163) and *Pharbitis nil* (85), dormant buds do not respond. Salisbury (163) cut away the apical meristem of *Xanthium* and applied auxin (NAA) to the cut surface. The treated plants flowered, the untreated ones did not; therefore, it was concluded that auxin overcomes the requirement for active buds. (Possibly auxin activated the dormant buds in time to react to the floral stimulus before it had become dissipated.) Conversely, vigorously growing apices may also be insensitive to the floral stimulus (viz., in plants that never form a terminal inflorescence) or react less readily to it, as is the case in, e.g., *Glycine max* (44) and *Pharbitis nil* (79). In the case of soybean, reducing growth by inverting the plants promoted flowering which occurred earlier and at lower nodes (45). On the other hand, removal of the apex, allowing vegetative growth of axillary buds, delayed flowering of the latter. The fact that removal of young leaves and, in the case of inverted plants, of suckers resulted in earlier flowering, points to the existence of a correlative inhibition; in the research under discussion, this inhibition was ascribed to auxin. Application of nicotine sulfate, which in several ways acted as an antiauxin on soybean, also promoted flowering (44, 45, 46).

In conclusion it should be noted that various schemes have been put forward in recent years to account for the reactions of SDP (e.g., 172, 173, 200). Although these are of considerable interest, a discussion will be omitted here because it would require several pages.

LONG-DAY PLANTS

The light process.—Since the light process and the dark process closely interact a separate treatment is only justified for convenience.

It is generally accepted that the light perception takes place in the leaves. Cajlahjan (25), however, is of the opinion that photoperiodic induction in LDP which form rosettes in short day, as for instance *Rudbeckia*, is im-

possible in plants without roots. Lang [(105), p. 455] opposes this view on the basis of unpublished results of Liverman and Lang with *Hyoscyamus niger* and *Silene armeria*.

The literature on the light process centers around the wavelength of the light. Stolwijk (181) treated cruciferous plants with a basic illumination of white light, followed by supplementary light of well-defined spectral regions. *Brassica rapa oleifera annua*, *Sinapis alba*, and *Iberis coronaria* were found to be senstive to blue and infrared, and insensitive to the region 5200 to 7000 A. Flowering in short days was possible in blue or infrared, not in white light, where the region 5200 to 7000 A inhibits the flowering effect of the blue component of white light. On the other hand, *Cosmos bipinnatus* and *Spinacia oleracea* showed reactions exactly opposite: i.e., sensitive to 5200 to 7000 A and with little or no response to blue and infrared.

The wavelength dependency of *Hyoscyamus niger*, as studied by Stolwijk & Zeevaart (182) is complex. *H. niger* is a qualitative LDP, so that flowering in short days does not take place. The photoperiodic reaction takes place by extending white short days with light of any spectral region. The high-intensity light reaction takes place in exclusively violet, blue, or near infrared light, not at all in green or yellow, while red has only a slight effect. Red light does not influence the action of violet, blue or infrared light. Infrared is active as supplementary light of low intensity; however, red is not and there is no antagonism. This behaviour is the same as in the above-mentioned Cruciferae. On the other hand, when given as high-intensity night interruptions, red is most active, while infrared is antagonistic.

Kandeler (89, 90) established that one strain of *Lemna gibba* is an LDP. The critical daylength is 12 to 14 hr. at 30°C., but 10 to 12 hr. in water-filtered light, for which difference the absence of far infrared of $>15{,}000$ A is responsible [(89), p. 161]. *L. gibba* is interesting both from a morphological and from a physiological standpoint. Flower differentiation does not involve the terminal and the lateral growing points, so that vegetative and generative growth can take place side by side. As to wavelength dependency, *L. gibba* reacts favourably to supplementary light of all regions of visible light, but there is a maximum response in infrared of 7200 to 7600 A. Weak red light of 6000 to 7000 A favours flower formation, but high-intensity red light inhibits it. The sensitivity to periodically-applied additional light during the dark phase (partly interrupting light) decreases rapidly from the beginning to the end of the dark phase.

It has been considered as a general rule [(104), p. 281] that red light is the most active source of supplementary light, with the exception of cruciferous plants. It seems that *H. niger* and *L. gibba* belong to the exceptional group, although in other respects they differ from each other. The importance of the light source has been demonstrated in *Silene armeria* by Takimoto (185), who found Mazda incandescent 20-watt lamps effective, but Mazda daylight fluorescent 20-watt tubes ineffective. The absence of long wavelengths, perhaps infrared, in the latter is responsible.

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The dark process.—If in LDP darkness only inhibits, flowering under the critical daylength must take place when the dark process is stopped. This can be done by applying low temperature. *Arabidopsis thaliana* is an LDP with an exceedingly low critical daylength of 4 hr. for very early strains, 5 hr. for late strains. Laibach (102) could induce flowering in the latter with cycles of 4 hr. of light at 23°C. and 20 hr. of darkness at slightly more than 0°C. Oleinikova (144) found that low temperature during the dark phase increases flowering in *Triticum*, *Hordeum*, and *Avena*. Zdanova (222) applied a nitrogen atmosphere during the nyctophase which increased the flowering of *Rudbeckia bicolor*. One of the present authors obtained preliminary unpublished results with *Trifolium pratense* which completely oppose the concept of dark inhibition. A treatment of 16 hr. light and 8 hr. darkness at greenhouse temperature induced flowering, while at 5°C. the process was completely stopped. With a warm photophase and a cold nyctophase the process was also stopped, but with a cold photophase and a warm nyctophase induction started. These results have to be confirmed before their meaning can be discussed.

It is well known that LDP, grown in short-day conditions, initiate flowers when the dark period is interrupted with a brief exposure to light. For *Hordeum vulgare*, var. "Wintex" and *Hyoscyamus niger*, Downs (42) found that the red-infrared antagonism (discussed in detail on pp. 149–154 above) is active. In "Wintex" barley, red promotes flowering, while infrared does not reduce the flowering effect of red, but slows down the floral development considerably. In *H. niger* also the flowering is greatly reduced. These results agree with those of Stolwijk & Zeevaart (182) in *H. niger* which already have been discussed.

The flowering of defoliated *H. niger* in complete darkness has been mentioned by Lang (104). Such flowering seems most unexpected at first sight. Fife & Price (43) established that well-developed sugar beet roots developed seed stalks during a long dark period at a temperature of 5°C. Also, after a long dark cold treatment seed stalks developed in darkness at 15°C. to 28°C. The flowering was much better in long days and seed setting did not occur in darkness. Hence, light is not essential for flower production, provided enough storage material is present. Sugino (183) grew a very early spring wheat on White's agar medium with sucrose in continuous light (CL), in short days or in darkness, and obtained flower initiation in all three cases. Earliest initiation occurred in CL and plants in short days were earlier than those in darkness. Sucrose was unnecessary in CL, 2 per cent was needed in short days and 4 per cent in darkness. Analogous cases are *Pisum*, *Raphanus* and *Baeria*, in which, however, short days give the slowest development. These results fit into our concept of leaves inhibiting during the dark phase, only if we accept the theory that the inhibiting function takes place only under the effect of the light phase.

Relative length of photo- and nyctoperiod.—Under natural conditions the relative duration of the light and of the dark period plays an important part

in flower initiation. Insight into this light:dark ratio can be obtained by changing the natural 24 hr. cycle. Takimoto (184) has studied the flowering response of *Silene armeria* to thirty combinations of 4 to 24 hr. light with 4 to 36 hr. darkness. With constant dark periods and various light periods flower initiation increases as the light period increases. The same holds true with constant light periods and decreasing dark periods. With <12 hr. light, floral induction does not occur with 14 or more hr. darkness, but with 14 or more hr. light, floral initiation takes place with 24 hr. or more darkness. The extreme treatments in the last case have been 14 hr. light plus 24 hr. darkness and 24 hr. light plus 36 hr. darkness. With <12 hr. light, the dark period is determining, but with 14 hr. or more light, the light period is determining. The dark period tends to destroy the effect of a light period, unless the latter has been "fixed" after relatively much light. Lang [(108), p. 318] commented upon this last conclusion and prefers Lang and Melchers' idea that light has no direct effect upon flower initiation, but acts merely as absence of darkness: as, for example when in *H. niger* the leaves (in which the dark inhibition is located) are removed, flowering occurs in total darkness. The problem is whether darkness destroys a light effect, or light destroys a darkness effect, or perhaps both. It seems as if the last alternative cannot be neglected for the moment. It is possible that in *S. armeria* and in *H. niger* different mechanisms occur. A comparison of these species under identical circumstances would be interesting. Investigations of Laibach (102), with *Arabidopsis thaliana* published in 1951, tend to support Takimoto's conclusion. When the daylength decreases from 24 hr. to 8 hr., the necessary amount of light remains practically the same, but from 8 hr. to 4 hr. the light requirement increases, meaning that with relatively long days the dark period has no effect, while with decreasing daylength the dark period inhibits more and more.

Persistence of photoperiodic induction.—A great variability exists in the behaviour of LDP which are brought from inducing long days, or CL to short days. Chouard (35) established that *Anagallis arvensis* immediately reverts to the vegetative condition. In *Scabiosa ukranica* and *S. canescens* (=*suaveolens*) the reversion takes place slowly, while *Helianthemum guttatum* and *Nigella damascena* do not revert at all. *Arabidopsis thaliana* evidently belongs to the rapidly reverting type (102).

Factors influencing the induction.—Internal and external conditions, apart from daylength, may influence the photoperiodic process. Age plays a role and in certain cases a clear "juvenile phase" has been established. Laibach (102) did not find any influence of age in *Arabidopsis thaliana*, however, Chouard (36) found that *Stenactis annua*=*Erigeron ramosum* is an obligate LDP at low temperature, but an almost day-neutral plant at high temperatures. El Hinnawy (74) studied extensively the influence of mineral nutrition in the LDP *Sinapis alba*, *Anethum graveolens* and *Spinacia oleracea* just as in the SDP *Perilla crispa* and *Kalanchoë blossfeldiana*. The photoperiodic reaction was not principally altered by mineral nutrition, only modified. Flower initiation in *Sinapis alba* was favoured by very low N-levels. In the other

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plants studied initiation was practically unaffected. However, flowering behaviour could be greatly influenced not only by N, but also especially by Ca and P. The indirect effects on flowering are considered to be due to the production of building material for flower formation, and to the production of auxin necessary for growth. These results tally well with the existing ideas.

Growth regulators: auxins.—Auxin is supposed to influence induction in LDP favourably, but direct evidence is scarce. Recent work directs itself to the natural auxin levels and to direct application of auxin. With reference to the latter topic, Liverman & Lang (124) induced annual *Hyoscyamus niger* and *Silene armeria* to flower by daily application of IAA. The plants were grown under threshold conditions of 8 hr. high-intensity light and 16 hr. of supplementary light of different intensities. The controls remained vegetative. This case is said to give the first evidence of a qualitative auxin effect on an LDP. Simultaneously, Konishi [(98), p. 16, footnote] found that spraying 50 mg./l. IAA every day for about two months promoted floral initiation and bolting in *S. armeria*. The daylength has not been mentioned.

Konishi also determined the natural auxin level. In *S. armeria* (97) much more auxin is formed and less of it is destroyed in bolting plants than in rosettes. In an extensive and detailed study (98) the complete auxin metabolism (production, destruction, and inhibition of destruction) was studied in *S. armeria*, *Rudbeckia bicolor*, and *Spinacia oleracea*. In rosette plants and at the beginning of bolting, little auxin was found, either because the IAA-oxidizing destruction overcomes production, or production is equal or inferior to consumption. In bolting plants there is much auxin because of high auxin production and little auxin destruction due to IAA oxidase-inhibitor. Immediately before flowering there is much auxin: i.e., high production and little consumption due to decreased growth rate. Konishi tends to ascribe flower induction in LDP completely to auxin: "Bolting and flowering seem to be explained even without some unknown causal factors induced by long day effects" [(98), p. 59]. This is an interesting suggestion and the experimental evidence seems satisfactory. Marcelle Gilson (49), however, found the same amounts of auxin in plants of *Hyoscyamus niger* grown in 16 hr. as in 8 hr. daylengths. The difference in the auxin contents between flowering and nonflowering plants was considered by Gilson as an effect of sexualization and not its cause. Liverman and Lang's work (see above) received strong comment. Konishi's results were not known yet to Gilson.

The total evidence available points to auxin as a very important factor in the flowering of LDP, but before a general conclusion is justified, much more work must be done.

Growth regulators: gibberellins.—In 1956, Lang (109, 110, 162) reported bolting and flowering of nonvernalized *Hyoscyamus niger* after application of Ga. Since then Ga has been successfully applied not only to other cold-requiring plants but also to a great number of LDP under noninductive conditions (22, 23, 37, 111, 112, 122, 129, 130, 132, 133, 209, 210). However, not all LDP show a similar reaction. Many species elongate but do not flower, and

some do not even bolt (37, 129, 130). In some plants, e.g., *Digitalis purpurea* (122), Ga substitutes for both cold and long day; in others, e.g., *Oenothera biennis* (37) it substitutes for the thermal but not for the photoperiodic requirement. With the exception of certain Crassulaceae (21), all plants in which Ga promotes floral induction are rosette plants.

There are some topics of general importance arising from the work with Ga. In 1951 Mitchell *et al.* (138) extracted substances from bean seeds which must have been Ga-like. This was confirmed by Phinney *et al.* (149) and Bünsow *et al.* (24), while Radley (150) extracted active substances from pea plants, and Lona (131) from *Brassica napus oleifera*. Lang *et al.* (114) found that the endosperm of *Echinocystis macrocarpa*, either directly or extracted, concentrated or diluted, induces *H. niger* and *Samolus parviflorus* to flower. Hence there is complete evidence that Ga (or related substances) occur in higher plants. The question then arises what these natural gibberellins have to do with flowering. Lang (112) has concluded that Ga cannot be the flowering stimulus, but that it probably acts indirectly as a factor in the production of the flowering stimulus by causing the plant to bolt. However, this would imply that bolting plants always initiate flower buds and the above cited cases of "failures" clearly demonstrate that this does not hold true. Wellensiek (204, 205) has stressed the fact that a number of LDP react to Ga by flowering in short days while others bolt, but do not flower. He concluded that bolting and flower formation depend, at least in part, on different factors. Also, the mechanism of flower formation in the reacting LDP and in the nonreacting LDP cannot be the same, just as this mechanism in the nonreacting SDP and the reacting SDP cannot be the same. This makes unacceptable the idea that one universal flower hormone exists. In this respect it is noteworthy that Lang (112), who is a strong advocate of the hormone theory, hints at the possibility that more than one factor may be limiting. It will be quite interesting to see how our theoretical insight into the physiology of flowering will develop in the near future under the influence of further results with Ga and auxins.

Biochemistry.—In a very extensive and detailed study, Sironval (176) has reported on his work with *Fragaria vesca semperflorens*. This plant has a day-neutral juvenile phase, a long-day inductive phase, and an almost day-neutral sexual phase. These phases are irreversible and are morphologically well characterized by the number of leaf dents, physiologically by the chlorophyll metabolism. In short days the total amount of chlorophyll is relatively small and the ratio of chlorophyll *a*:*b* is high. In long days this ratio is the opposite. Unsaponifiable extracts from leaves of flowering plants induce a slight flowering response in young plants. Active elements in these extracts are sterols in high concentrations and vitamin E in low concentrations. A causal relation exists which can be summarized as follows: long day→chlorophyll metabolism→vitamin E (and other substances)→flowering. These viewpoints are certainly interesting, but a number of questions arise. (*a*) The young plants were induced to flower under inductive circumstances. Did

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the applied extracts shorten the juvenile phase and hence not act directly on flower initiation? (*b*) Clauss and Rau (39) found that the chlorophyll content in *Hyoscyamus niger* is minimal when flowering is maximal. This is just the opposite of Sironval's *Fragaria*. (*c*) Before the exact value of Sironval's extracts can be judged, they must be tested under noninductive circumstances and with more species.

Discussion.—We concluded that the mechanisms of the physiology in different SDP have much in common. It seems that among LDP a greater variability exists. This is demonstrated by the wavelength dependence of Cruciferae, *H. niger*, *L. gibba* in comparison with each other and with other LDP, but especially by the reaction to gibberellins. The flower hormone hypothesis is much less evident in LDP than in SDP.

We can watch the development of our knowledge with regard to auxins with much interest. There is a trend, indicating that they may play an important role, perhaps in certain plants more than in others. Also, further work with Sironval's extracts is looked for with impatience. But even more "elementary" topics, as the relation between photophase and nyctophase, cannot be considered as solved. We should not be surprised if further studies on this subject would clarify the relations between SDP and LDP.

THE RELATION BETWEEN SDP AND LDP

Plants Requiring Both Long Days and Short Days

In 1952, Resende (151, 152) found that certain Crassulaceae are "long-short-day plants" flowering neither in continuous long days nor in continuous short days, but only after having been transferred from long days to short days. The critical daylengths of the two phases apparently overlap a little, for *Bryophyllum daigremontianum* shows a weak flowering response when kept continuously in 12 hr. days (154). Gibberellin causes *Bryophyllum* to flower in continuous short days, but not in continuous long days, which shows that it substitutes for long days, but not for short days (21). A recent addition to the group of the long-short-day plants is the shrub *Cestrum nocturnum*, studied in some detail by Sachs (159, 160, 161). This plant requires a minimum of five long days followed by at least two short day cycles to flower. The long- and short-day cycles should be given in this order to the same leaf, which suggests that the product of long days is not translocated. The strength of induction, as measured by the number of nodes flowering per plant, depends not only upon the length of the short-day treatment but also on the intensity of long-day induction. This led to the hypothesis that the product formed in long days is a precursor of the short-day product. There is some evidence that both are heat labile. Phototemperature has the greatest effect upon long-day induction and the night temperature primarily affects the short-day process, which is in accordance with what is known about the inductive reactions in LDP and SDP. For long-day induction, a photoperiod of 16 hr. is superior to one of 24 hr., which suggests that long days, apart from their promotive

effect, may also have an inhibitory effect upon subsequent short-day induction. In the light of what is known about SDP this does not come as a surprise. Sachs suggests that the results of his work with *C. nocturnum* give a clue to the problem of the relation between SDP and LDP and advances the following hypothesis: in SDP the immobile precursor is formed independent of daylength, but its transformation into a transmissible floral stimulus requires short days; in LDP, formation of the precursor requires long days but the next step has no special daylength requirement.

Grafting Experiments

Evidence that the floral stimulus is the same in SDP and LDP is furnished by grafting experiments; an SDP may cause an LDP to flower in short days, and conversely, an LDP may induce flowering in an SDP in long days. Khudairi & Lang (95) have repeated and extended previous results (104) and shown that induced scions of the LDP *Hyoscyamus niger* (annual strain) and *Nicotiana sylvestris* caused the SDP *Nicotiana tabacum* "Maryland Mammoth" to flower in long days. *Xanthium canadense* flowered in long days when grafted onto the LDP *Erigeron annuus*, *Rudbeckia bicolor*, or *Centaurea cyanus* (143). The SDP *Kalanchoë blossfeldiana* has been induced to flower in long days by grafting with the LDP *Sedum kamtschaticum* (33), *S. ellacombianum*, and *S. spectabile* (220, 221). Conversely, the latter two LDP have been induced to flower in short days by grafting onto *K. blossfeldiana* (220, 221). *Bryophyllum daigremontianum*, a long-short-day plant, flowered in continuous long days after grafting with short-day species of *Kalanchoë* (153, 221). Similar results had been obtained previously by using the SDP Maryland Mammoth tobacco as a donor for *H. niger* and *N. sylvestris* in short-day conditions (104); these results have been confirmed (94). A fact brought out by these experiments is that the SDP used as donors cause flowering of the LDP only if the former are induced to flower themselves. A SDP, kept continuously in long day and consequently vegetative, does not act as donor of the floral stimulus to a scion of an LDP, kept in short day (221). This does not necessarily mean that synthesis of the floral stimulus proceeds along different pathways in SDP and LDP. One may apply Sachs' hypothesis (159) that the process is the same, but that the step that requires long days in LDP leads to a product that is not transmissible.

Endogenous Rhythms

Bünning and his school (10, 14, 16) regard photoperiodic responses as the manifestation of an endogenous rhythm interacting with the environmental rhythm. According to this theory, the plant passes daily through a photophile phase during which light promotes flowering (and other processes affected by light) and a skotophile phase characterized by an inhibiting effect of light. The rhythmical alternation of these phases is set into motion by the onset of the daily photoperiod, but is otherwise endogenous and not affected by the onset of darkness or by brief periods of light during the dark period. Leaf

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movements are looked upon as the external reflections of this rhythm and are used to measure its progress. The way in which this theory accounts for the difference between SDP and LDP is as follows. In SDP, the photophile phase begins immediately after the onset of the daily light period, and consequently the skotophile phase falls during the dark period, at least in short-day cycles. In LDP on the other hand, the photophile phase does not begin until about 12 hr. after the onset of light; consequently, unless the days are very long, the photophile phase will largely coincide with the dark period (10).

A judgment on the value of this hypothesis should be preceded by a discussion of the more fundamental aspects of the theory, viz., that light and dark stimuli do not act directly on the system leading to flowering, but intervene with a fundamental phenomenon that regulates many processes besides flowering, and that this phenomenon follows a diurnal rhythm which, although regulated by the environment, is basically endogenous. To start with the latter postulate, the reviewers give as their opinion that one may only call a rhythm endogenous when it can be shown (*a*) that plants show a preference for certain environmental rhythms, especially for 24-hr. cycles, and do not adapt themselves readily to cycles of arbitrary length, and (*b*) that the rhythmic phenomena also exist under constant conditions.

Evidence to support condition (*a*) has been presented by Blaney & Hamner (6) who subjected plants of Biloxi soybean to different light and dark periods and found that optimal flowering occurred when the cycles were 24 hr. or about 48 hr. long. Condition (*b*) can be tested by interrupting a long dark period by short light breaks. If the plant continues to pass through photophile and skotophile phases, the effect of the light break should depend on the time at which it is given and reflect the endogenous condition of the plant. Carr (28) grew plants of *Kalanchoë blossfeldiana* on 72 hr. cycles consisting of a light period of $11\frac{1}{2}$ hr. and a dark period of $61\frac{1}{2}$ hr. interrupted at various intervals by a light break of 1 hr. The inhibitory effect of this light interruption showed three optima, viz., at 6, 30, and 54 hr. after the beginning of darkness. These results were confirmed by Schwabe (171) but in his experiments the second maximum in the middle of the dark period was very slight.

Other data, however, gave no indications for the existence of an endogenous rhythm during long dark periods. Wareing (199) grew plants of Biloxi soybean on 48 or 60 hr. cycles, consisting of 9 hr. of light and 39 or 51 hr. of darkness, respectively. The dark periods were interrupted at various times by 30 min. of light. In both cases, the light break exercised its optimal inhibitory effect on flowering when given 6 hr. after the beginning of the dark period or 6 hr. before the end of it. When occurring in the middle of the dark period, it promoted flowering. In *Xanthium* a light break given at the beginning of a long dark period inhibited flowering, but one given towards the end had slight or no effect.

Kribben (100) who worked with the SDP *Coleus blumei*×*fredericii* found no inhibitory effect of light in the middle of a $60\frac{1}{2}$ hr. dark period; a light inter-

ruption depressed the flowering response only when given at the beginning or near the end of a dark period. Similar experiments have been done with LDP. In *Hyoscyamus niger* the flower-promoting effect of a light interruption showed three optima in the course of the dark period, but the second optimum was much less pronounced than the first and the third (39). With *Anagallis arvensis*, light breaks given at the beginning or towards the end of a 60 hr. dark period promoted flowering, but one given in the middle had no effect (78).

It is fortunate that Wareing's experiments have been repeated and extended by Bünning (12, 13) to show how the results could be fitted into his concept of the relation between photoperiodism and endogenous rhythms. From a study of leaf movements he concluded that in Biloxi soybean the rhythm comes to a standstill after 30 hr. in continuous darkness. A light break given after this time has the effect of setting the rhythm in motion again, so that it is now the position of the following main light period which determines the degree of flowering (in other words, the functions of the light break and the main light period have been reversed). Leaf movement studies in *Xanthium* gave a completely different picture. In this plant the rhythmic movements were found to be regulated by the beginning of the dark period instead of the light period. This regulation is such that the cycle length of internal and environmental rhythm are the same whether the cycle is 24, 32, or 18 hr. long [(13), Fig.1], which can only lead to the conclusion that *Xanthium* has no endogenous rhythm.

A fact that these researches again bring out is the close correlation between leaf movements and the sensitivity to light stimuli with regard to the flowering response. In Biloxi soybean, maximal rise of the leaves coincides with optimal promotive effect of light on flowering, while maximal declination coincides with optimal inhibitory effect. Leaf movements are much less pronounced in day-neutral varieties of soybean. In *Xanthium* a similar correlation exists as in Biloxi soybean, but in this plant the coincidence of leaf declination and effect of light on flowering is just the reverse (13). In *Kalanchoë blossfeldiana* the leaves are fixed but the petals show rhythmic movements, which according to Bünsow (20) are correlated with the flowering response of the plant. Schwemmle (174) found a correlation between phase of petal movement and reaction to a short period of high temperature. All these observations support the notion that leaf or petal movements and fluctuations in sensitivity to light and temperature all reflect an endogenous rhythmic process. However, they do not constitute proof for this hypothesis as coincidence does not necessarily mean a causal relation. In this connection one is reminded of *Phaseolus multiflorus*, a classical plant for the study of leaf movements but insensitive to daylength with regard to flowering. More damaging to the hypothesis is the fact that in 14 hr. days the SDP *Coleus fredericii*, the LDP *C. blumei* and their hybrid, SDP, show principally the same leaf movements (100). As discussed elsewhere in this review, many plants, SDP as well as LDP, flower in continuous darkness, where the rhythm

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has been shown to come to a standstill, while LDP flower in continuous light, where presumably something similar happens.

Although one of the merits of Bünning's theory is that it suggests an explanation of the difference between SDP and LDP, there is little experimental evidence on this aspect. In the LDP *Anethum graveolens* it was found that darkness given 9 to 11 hr. after the onset of the light period inhibited flowering to some extent, but a dark period given 3 to 5 hr. after the beginning of the photoperiod promoted it. From this it was concluded that *Anethum* is in the skotophile phase directly after the onset of light and passes into the photophile phase after about 10 hr. of photoperiod (17). This is the only case found so far that is in accordance with the theory as originally postulated. *Madia elegans* shows two photophile phases per day, separated by a short skotophile phase about 12 hr. after the onset of light (11). *Spinacia oleracea* and *Plantago lanceolata* are indifferent during the early hours of day but become photophile after about 6 hr. of light (17). Some evidence for a diurnal rhythm was found in experiments on the effect of a light break during a long dark period on flowering of *Hyoscyamus niger* (39) but similar experiments with *Anagallis arvensis* and *Arabidopsis thaliana* yielded negative results (39, 78).

Much work has been done to elucidate the nature of the fundamental process supposed to underlie the rhythmic phenomena. Fluctuations in enzyme activity and metabolism have drawn much attention, but as Bünning remarks in his review (14) it remains in doubt whether these are causes or consequences. More recently it has been shown that the poisons, colchicine, urethan and phenylurethan, which are known to affect the nuclei, can completely upset the diurnal rhythm (15). This led to the discovery of rhythmic changes in the size of the nuclei in various tissues. From these data, Bünning *et al.* drew the conclusion that structural changes of the nuclei are an element of crucial importance in the mechanism of the endogenous rhythm (19).

In retrospect, the necessity of accepting the theory of an endogenous rhythm to explain photoperiodic responses with regard to flowering does not seem to be very great yet. In most cases "a direct action of light and dark stimuli on the system leading to flowering provides a simpler hypothesis" [Schwabe (171), p. 276]. In the few cases where acceptance of an endogenous diurnal rhythm seems unavoidable, its effect on the photoperiodic response appears to be slight. It remains possible that one is dealing with two responses to external stimuli: one that affects flowering directly, and another, much weaker one, that affects it via a system subjected to an endogenous rhythm.

PHOTOPERIODISM AND VERNALIZATION

The subject of vernalization falls outside the scope of this review, but something has to be said about it as some evidence suggests a close relation between the flowering responses to low temperature and to the photoperiod.

In the first place, there seems to be a correlation between the requirement of low temperature and of subsequent daylength for optimal flowering. All plants that can be vernalized by subjecting the seed to low temperature are LDP, and plants that can only be vernalized after having reached a certain size are either LDP or day-neutral plants [with one exception: *Chrysanthemum morifolium*, which is an SDP (167)]. Melchers (136) expressed disbelief in a causal relation between the requirements of low temperature and long day. In his opinion, both requirements are caused by separate genes, which occur in this combination because of the selective influence of the temperate climate, where winters are characterized by cold, and spring by long days. However, a physiological connection has now been suggested by the effect of gibberellins which may substitute for both cold and long day. This aspect has already been discussed.

Cajlahjan *et al.* (26) showed that unvernalized scions of biennial *Brassica campestris* could be induced to flower by grafting on vernalized or annual *B. campestris* or *B. oleracea*, but only in long day and with leaves on the stock. They suggest that the vernalized condition of the apical meristem is transmitted to the newly formed leaves, enabling these to react to the inductive daylength. A similar hypothesis has been proposed by Wellensiek *et al.* (207). The concept of a vernalized condition transmitted by cell division suggests a role of the proteins. However, Aach & Melchers (1) who studied the effect of low temperature on the nature of the proteins by serological methods, could not establish a correlation between the differences found and the vernalized condition.

The photoperiod preceding the cold treatment may influence the effectiveness of the latter. Junges (88) found that optimal flowering of kohlrabi (*Brassica oleracea gongylodes*) occurs when vernalization is preceded by a period of long days and high-light intensity followed by short days. Flowering of *Beta vulgaris* is slightly promoted when the cold treatment is preceded by short days. A fact that has a more direct bearing on the relation between vernalization and photoperiodism is that in *Campanula medium* short days can substitute for low temperature. The response to either stimulus is qualitative, so that a plant which has not been subjected to either low temperature or short days (and subsequently to long days) remains vegetative (203). Until now only one similar case was known, viz., *Scabiosa succisa* (37). In rye, short days may also substitute for low temperature but in this case the responses are only quantitative (51). In *Chrysanthemum* a limited period of long days given immediately before or after vernalization hastens inflorescence initiation, but the cold requirement itself is not affected (168). Conversely, vernalization may substitute, to some extent, for the photoperiodic requirement. In radish, unvernalized plants flower only in long days, but vernalized ones also in short days and continuous darkness (187). In spinach, vernalization shortens the critical daylength from 14 to 8 hr. (195).

Most of the available evidence suggests that low temperature is perceived by the growing point (104, 169). However, this may not be generally true.

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Hagemann (59) made leaf cuttings from flowering (vernalized) and vegetative (unvernalized) plants of *Lunaria biennis*. He obtained flowering plants from the former, vegetative plants from the latter, which suggests that the induced state not only prevailed in the growing point but also in the leaves. It is not clear whether the leaves used as cuttings had been formed by the growing point before or after it had been vernalized. Oehlkers (141, 142) studied floral induction in a unifoliate species and a similar hybrid of *Streptocarpus*, both requiring low temperature (10 to 15°C.) to flower. He established that the temperature is perceived by the leaf; the embryo cannot be vernalized. After vernalization the leaf was cut into small fragments from which new plants could be regenerated. Some of these flowered without further vernalization, others did not, depending on the original position on the leaf of the fragment from which they had developed. From a detailed study of the pattern ensuing after different cold treatments, it was concluded that a floral stimulus makes its appearance in the middle of the leaf as a result of low temperature treatment and from there it is transported towards the base. Hess (72), using paper chromatography, studied the auxin content of leaves of *Streptocarpus wendlandii* during induction at 12°C. His results permit the hypothesis that a high auxin content inhibits the formation of the floral stimulus.

The experimental results summarized could inspire a long discussion, but, as this would be very speculative it will be omitted. The main source of uncertainty is the difficulty in giving a physiological definition of vernalization. Temperature affects every physiological process in the plant and it is hard to tell whether low temperature acts on the same process in *Triticum*, *Brassica*, and *Streptocarpus*, for example, although in each of these cases the final result is flower induction.

DAY-NEUTRAL PLANTS

Although the majority of species is day-neutral, that is, indifferent to the photoperiod, there is little work on this group, most of it limited to plants of economic importance and undertaken with a practical end in view. This work offers much of interest to the physiologist, but most of it is rather fragmentary and does not permit any definite conclusions. Therefore, an exhaustive review will not be attempted and only the evidence concerning some of the more intensively studied plants will be summarized.

Pisum sativum.—In most pea varieties flowering is promoted by long days, but the early ones are indifferent to daylength (208). Haupt (65) found that in "Kleine Rheinländerin" the first inflorescence is initiated five days after sowing at the ninth or tenth node. When the cotyledons are removed flower initiation is delayed and becomes dependent on the photoperiod: in 24-hr. days the first inflorescence appears at the eleventh node, but in shorter days a further delay occurs until in 4-hr. photoperiods the inflorescence is formed at the seventeenth or eighteenth node. In total darkness, however, it is formed at the twelfth or thirteenth node (69). This is in accordance with

a suggestion of Hänsel (58) that early varieties are potential LDP but that flower initiation occurs before the leaves have unfolded and can react to the photoperiod. Haupt (66) tried to extract the flower promoting agent from the cotyledons but succeeded only in obtaining substances which, when added to the nutrient medium, delayed flower formation. A similar inhibitory influence was obtained with yeast extract, casein hydrolysate and nitrate, and was shown to be due to nitrogen. This suggested that in this case the old theory of Klebs concerning the importance of the balance between carbohydrate and N-content might apply. However, it was found that lack of sugar in the nutrient medium promoted floral induction (although to a lesser extent than N-deficiency), so that the most rapid flower initiation was obtained at low levels of both sugar and nitrogen (67). Probably, the effect of these substances is, at least partly, indirect, viz., via an influence on the balance between vegetative and reproductive growth. When vegetative growth of young seedlings of the late variety "Alderman" was depressed by growing the shoot tips as cuttings, inflorescence initiation occurred at a lower node than in the intact control plants (68). Flowering in early peas is apparently controlled by a number of substances from the cotyledons: a non-extractable promotive agent, and sugar and nitrogen, which have an inhibitory effect on flowering caused, in part, by a stimulation of the vegetative activity of the plant. [It is not inconsistent with this view that, as Sprent & Barber (180) have shown, an inhibitory agent is removed from the seed by washing.] That the promotive agent present in the cotyledons of early peas is the same as that formed in the leaves in long days is suggested by two observations: (*a*) when the cotyledons are removed, day-neutral varieties become sensitive to daylength (65), (*b*) the long-day requirement of late varieties disappears when they are grafted on early ones (3, 147). Low temperature treatment accelerates subsequent flower initiation by about three to four nodes (73). Apparently the only pea varieties that can be vernalized are also LDP (208). The "Alaska" pea, which is day-neutral, does not respond to low temperature, unless, as Leopold & Guernsey (118, 119) have found, low temperature treatment follows an application of auxin (principally NAA). Auxin alone does not promote flowering. This effect, which they called chemical vernalization, was reduced by high intensities of light, and nullified by heat, nitrogen atmosphere, or CO_2-free air applied for some time after the cold treatment.

Ipomoea batatus.—Of the sweet potato a number of varieties are grown which rarely or never flower. Temperature and daylength have little or no effect on this behaviour, but flowering has been obtained by grafting onto nontuberous species of *Ipomoea* and related genera (92, 225). This success has been attributed to the fact that since no storage organs could be formed, more photosynthates became available for flower initiation (92). However, there are facts suggesting that the effect of the donor plant is more specific: (*a*) not all nontuberous *Ipomoea* stocks induce flowering in sweet potato scions; (*b*) in successful combinations the flowering response is very rapid,

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flowers appearing on the first node differentiated after grafting (77, 103); (*c*) flowering is only induced when leaves are left on the donor plant; (*d*) sweet potato stocks (with tubers) will flower when grafted with scions of morning glory, *Ipomoea purpurea* (77). Howell & Wittwer (76, 77) were able to induce flowering of certain sweet potato varieties by sprays of the Na-salt of 2,4-D. The optimal concentrations were surprisingly high, viz., 100 to 500 p.p.m., depending on the variety. Flowering was general and associated with, but not necessarily related to, injuries to leaves and stems and a significant depression of storage root growth.

Lycopersicum esculentum.—In the tomato the number of leaves to the first inflorescence is determined by temperature and light intensity (155, 193). Low temperature (10 to 12°C.) given during a period beginning at cotyledon expansion and ending approximately nine days later reduces the number of leaves to the first inflorescence. A cold treatment of the germinating seed had no effect (27, 211, 212). Under optimal conditions, plants initiate an inflorescence after eight or nine leaves. At lower light intensities initiation is delayed. Plants grown at an intensity of 200 μw./cm.^{2} died after having formed 24 leaves but no inflorescence (214). The position of the first inflorescence is not affected by auxin, nor by gibberellin. However, its appearance is hastened by applications of N-tolylphthalamic acid (189). Tri-iodobenzoic acid causes disorganized growth, removal of apical dominance and a shift in the balance between vegetative and generative growth, which leads to formation of inflorescences by all growing points, also by those in the axils of the cotyledons (50). Although mature tomato plants flower profusely under any conditions permitting growth, they do not act as donors for a floral stimulus in graft combinations. Shy-flowering potato varieties can be induced to flower profusely by grafting on tomato (190), but this is probably an effect on flower differentiation and development and not on floral induction. There is a great amount of literature on inflorescence differentiation and development in the tomato, some of it of considerable interest, but this falls outside the scope of this review.

Ananas comosus.—The pineapple plant normally initiates its flowers in November, under the influence of low night temperatures (146). When sprayed at this time with auxin, flowering is depressed; high concentrations of auxin inhibit it altogether (38). However, when auxin is applied earlier in the season, it induces flower initiation in plants that otherwise would remain vegetative. Various auxins are effective, principally NAA, 2,4-D and indolebutyric acid, but IAA is almost inactive. The theory has been advanced that under natural conditions flowering is brought about by the accumulation of auxin in the apical meristem. Van Overbeek & Cruzado (145) observed that a pineapple plant will flower when placed horizontally, which they ascribed to an accumulation of auxin at the underside. However, flowering is also brought about by ethylene, acetylene, and beta-hydroxyethylhydrazine (53); these compounds are neither auxins nor lead to an increase of auxin content (116). Gowing (52) has advanced the hypothesis that the synthetic

growth regulators act by competitively lowering the effective level of native auxin in the stem tip. Some evidence has been presented that IAA is the auxin of importance in pineapple flowering. Its ineffectiveness when applied to the plant is possibly due to the presence of IAA-destroying enzymes. Further literature concerning flowering of the pineapple has been reviewed by Leopold (116, 117).

Litchi chinensis.—In Hawaii the lichee is irregular in flowering. Flower initiation occurs usually in October and November, probably under the influence of low night temperatures. When there is heavy rainfall during autumn, premature growth of young vegetative shoots occurs and flower initiation is curtailed. Under these conditions, applications of the Na-salt of NAA promotes flowering, probably by preventing premature vegetative growth. As girdling of trunks also induces blossoming, Nakata (139) advances the hypothesis that a high carbohydrate level is required for flower induction in this plant.

Malus sylvestris.—Because of the size of the plant, its genetical heterozygosity, and the length of the juvenile phase, little progress has been made with the study of the factors determining the first flower induction of apple seedlings. More is known about recurrent flower initiation of adult trees. Differentiation of flower buds occurs during summer, starting at about the time vegetative growth ceases. Bending of branches, ringing of the bark and grafting on weak rootstocks lead to early cessation of growth, accumulation of carbohydrates in the aerial parts of the tree and abundant flowering. An adequate leaf area is required. The effect of these leaves is quite localized: removal of the two top leaves on a spur will prevent flower initiation (40). Fruits inhibit flower initiation not only in buds on the same spur but also on adjacent ones (95, 192). Consequently, fruit thinning promotes flower bud formation (2). There are as yet no indications of a specific floral stimulus, but there is evidence that flower initiation is associated with a high concentration of carbohydrates in the apical meristem and it is generally assumed that a causal relation exists (96, 223).

Discussion

It is tempting to regard day-neutral plants as plants in which the processes requiring long days in LDP and short days in SDP progress independently of daylength. This hypothesis is supported by the existence, within several species, of varieties forming a series ranging from either qualitative SDP or qualitative LDP to day-neutral plants, and by the possibility of inducing flowering in SDP or LDP under adverse conditions by grafting with day-neutral plants (104, 221). The actual state of affairs is more complicated, however. The case of *Pisum* shows that plants may be insensitive to daylength because a floral stimulus is present in the seed. This stimulus may be the same as that formed in the leaf so that in this case at least the inductive mechanism is comparable to that of LDP and SDP. In other cases, however, this is doubtful. The tomato can be grafted with many Solanaceae, but has

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never been shown unequivocally to induce flowering in the latter. The day-neutral *Nicotiana tabacum*, var. "Delcrest" does not induce flowering in long days of the SDP Maryland Mammouth tobacco, although it allows the stimulus to pass when used as an interstock (219, 221). The case is remarkable because both varieties belong to the same species, and may differ, as far as the flowering behaviour is concerned, in as little as one gene.

The striking effect of growth regulators on flowering of sweet potato and pineapple is as yet unexplained. Flower initiation of lichee, apple and other trees is apparently strongly affected by correlative processes in which auxins and carbohydrates play a role. Similar correlations have been found elsewhere, e.g., in the SDP jute (186) and *Glycine max* (44), but in the latter case they are dominated by a process involving a more specific floral stimulus. Whether this exists in fruit trees also is purely a matter of conjecture.

CONCLUSION

Although an effort has been made to include all relevant literature, a number of papers have been omitted for lack of space. These can be divided into two groups. First, much work has been undertaken to clarify the responses of plants of economic importance. The results have been omitted when they either confirmed results obtained with other plants, or were not detailed enough to be of much interest to the physiologist. Secondly, there are a number of studies on the correlation between floral induction and the content of proteins, amino acids, enzymes and other substances [e.g. (137, 178)] or, conversely, on the effect on flowering of substances known to poison enzyme systems. In so far as these papers have been left out, it has been because it is as yet hard to see how the results fit into the general picture of the physiology of flowering. However, as this picture is still quite fragmentary, some of the data omitted here may be considered to be of primary importance a few years hence. In any case, discoveries, such as the fact that certain types of virus have a striking effect on flowering (9, 48), are quite intriguing.

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THE LIGNINS[1]

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PURPOSE AND SCOPE

The purpose of this review on the lignins is to present recent literature from a standpoint which interests the plant physiologist and the biochemist. The period of time covered is from 1952 through 1957. This interval has been chosen because it marks a period of renewed interest in the biogenesis of lignin and because the preceding literature was well summarized in 1952. In addition, a number of reviews which concern either general or special questions about lignin have appeared in this interval. An effort has been made to incorporate pertinent viewpoints or the net conclusions of these reviews in this discussion, but duplication of coverage will be avoided.

The indices to original literature which have been used are: *Bibliography of Papermaking and U. S. Patents*, 1950–51, West; *Bibliography of Papermaking*, 1951–55, Weiner; *Bibliography of Papermaking and U. S. Patents*, 1955–56, Weiner; *Bulletin of the Institute of Paper Chemistry*, Library Notes, 1957, Vol. 27, No. 5, to Vol. 28, No. 4 incl.: *Chemical Abstracts*, 1950 through 1957; and *Biological Abstracts*, 1952 through 1957.[2]

The following titles have been selected to indicate the scope of recent interest in lignins. The reader is referred to them as a guide to the many ramifications of lignin research to be found in the literature.

1952. *The Chemistry of Lignin* (1); *Structure of Lignin, Reactions and Synthesis in Plants* (2).
1953. *Unsolved Problems of Lignin Chemistry* (3).
1954. *The Effect of the Extraction of Wood on the Composition of Isolated Lignins* (4).
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1957. *Lignification* (8); *Lignin-Carbohydrate Complex in Wood* (9); *Biochemistry of Lignin: Collected Papers, 3rd Lignin Round Table* (10); *Biological Decomposition of Lignin* (11).
1958. *Lignins and Lignification* (12).

DEFINITION

The use of the plural "The Lignins" as the title of this review is appropriate. There is a variety of lignins or ligninlike materials. To the botan-

[1] The survey of literature pertaining to this review was concluded May, 1958.

[2] The reviewer is grateful to his associates at The Institute of Paper Chemistry for many opportunities to discuss lignin questions. However, he is solely responsible for the text, both as to reporting the work of others and as to any expressed or implied comments.

ist lignin is an important metabolite of the growing plant, or, in a mature plant, it is a structural element which contributes strength and durability to perennials. The microbiologist and soil chemist deal with lignin as a residue of decay, ultimately as humus in the soil. To the organic chemist, lignin is a complex colloid which challenges his basic interest in chemical structure. It is a plant constituent to be broken down and then synthesized like other organic materials of whatever origin. The pulp and papermaker has still a different interest, the economical removal of lignin from fibers and the disposal of lignin-containing waste. At this point the stream biologist becomes involved and it may be said that the lignin cycle in industry is completed from biological synthesis to biological destruction.

To each investigator, lignin presents a different variation in its range of properties, a different set of problems. Quite likely it is a different, or at least a differing, material in the various situations in which we deal with it.

Whatever special viewpoint the investigator may have, a definition of lignin should proceed from its origin in plants. as in the following:

> The concept of lignin is derived first of all from its morphology. Lignin is a substance which permeates the membranous polysaccharides and the spaces between the cells, thereby strengthening them. Its presence brings about a physiological death of the tissue. It is a functional component of wood and it occurs in mature wood as a preformed, completed substance.

Many attempts have been made to define lignin in terms of its chemical structure, but the best that can be said is that "the chemical characterization of lignin proceeds from an idealized picture for which spruce lignin is the prototype" (13). In this sense, lignin is considered to be a colloidal, thermoplastic polymer derived from coniferyl alcohol. It is insoluble in water, in most organic solvents, and in strong sulfuric acid. It gives a characteristic, red-violet color reaction with phloroglucin and hydrochloric acid. It has a variable elementary composition and methoxyl content. It reacts readily with sodium bisulfite or thioglycolic acid to form soluble products. Its ultraviolet spectrum indicates the presence of aromatic ring structures and it yields up to 25 per cent vanillin under suitable conditions of oxidation. Hardwood lignins differ from the spruce lignin prototype by reacting positively (red color) to the Mäule test (chlorine oxidation, then sodium sulfite) (14). Correspondingly, syringaldehyde is found together with vanillin in the alkaline nitrobenzene oxidation products, frequently in equal or larger amounts than the latter. The formation of *p*-hydroxybenzaldehyde is, to a degree, characteristic of the oxidation of lignin from monocotyledons (15).

Efforts to refine the chemical definitions of lignin have resulted only in a still greater perplexity. A recently proposed set of "lignin criteria" comprehended eight categories of chemical reactions or physical measurements, many of them complex, performed preferably both with the isolated lignin and the wood from which it was derived. Even then "lignin could not clearly be defined" (16).

LIGNIFICATION

Morphology

Since the best definition we have of lignin is based on its morphology, it is appropriate to consider the process of lignification first from the standpoint of the sequence of events in the plant. The development of mature wood-fiber has been arbitrarily divided into four phases: (*a*) cell division, (*b*) cell enlargement, (*c*) cell wall thickening, and (*d*) lignification. These are not separate and distinct stages. "To appreciate the biological significance of lignification, it must be regarded as part of an ontogenetic sequence, i.e., not simply as a chemical change proceeding in the cell wall; it is intimately related to the changes in form and dimension by which the developing cell acquires its cytological identity" (17). It has been known for some time that lignification is heaviest in the middle lamella and the primary wall of wood fibers. More recently it has been shown that, in cross sections of *Pinus radiata*, the first evidence of lignification was to be seen in the primary walls adjacent to the corner thickening of the intercellular substance. From these beginnings lignification extended to the intercellular layer and primary wall generally and subsequently into the radial walls. It was noted that the initiation of the process was confined to individual cells. The deposition of cellulose and other cell wall carbohydrates appeared always to take place before the deposition of lignin. In longitudinal sections, lignification began near the middle of the cell and proceeded towards the tips (18).

Lignification, however, did not follow this pattern in primary wood, i.e., wood formed near the growing tips in either *Pinus radiata* or *Eucalyptus goniocalyx*. In these cells the lignin was deposited in the secondary layers of the cell wall and not in the primary wall or intercellular layers (19).

Morphological evidence showed that the process of lignification is affected by both internal and external influences. Internally, contact with parenchyma cells tended to delay the lignification of the adjacent walls of fiber cells. Applying an external force so as to bend a Eucalyptus sapling to one side greatly reduced lignification in those portions of the stem in which the cells developed under tension. Reversing the direction of the external force, i.e., bending the sapling in the opposite direction, caused lignification in cells which developed under compression, but did not induce it in those cells which had previously matured under tension (20).

Chemical Precursors

Almost all recent speculation about lignification has centered on coniferyl alcohol as the primary unit in a process of oxidation-polymerization. The prevailing concept is that spruce lignin is the prototype and that hardwood or other lignins are variants. Historically, this idea developed from three facts: the isolation of the glucoside coniferin from some conifers, the similarity in the elementary analyses of isolated lignin (Klason) and coniferyl alcohol, and the tendency of coniferyl alcohol to resinify. Recent experiments

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have shown that mushroom oxidases convert coniferyl alcohol into products which have ligninlike chemical properties and which range from dimers to polymers in molecular weight. The origin of coniferyl alcohol has been related to the carbohydrates by analogy to the pathway of microbial metabolism from sedoheptulose via shikimic and prephenic acids to phenylalanine and tyrosine. Taken as a whole, this scheme of lignification has much appeal because it refers many points in the process of lignification to biochemical reactions which are now considered in harmony with the generalities of plant metabolism (21).

However, it is probably premature to say that this scheme represents either the general pathway to lignification or that it is an actual pathway in any given species. Even among the conifers, instances of the actual isolation and identification of coniferin are few in comparison with the total number of species. In hardwoods the glucoside syringin is thought to parallel the function of coniferin. To date, there have been only 27 verified occurrences of syringin, practically limited to the olive family. In contrast, some compounds, specifically cinnamic acid and caffeic acid (perhaps in the form of chlorogenic acid), which may be biochemical equivalents of coniferyl and sinaphyl alcohols (respectively, their glucosides), have been reported from a much larger number of species and wider range of plant families (22).

Tracers

The availability of radioactive elements has led inevitably to many attempts to use them in the study of the lignins. These efforts can be grouped roughly as: (*a*) attempts to prove that coniferyl alcohol is a direct precursor of lignin; (*b*) attempts to prove that other compounds which fit into the current (Adler) scheme of lignin biosynthesis also participate in lignin formation; and (*c*) physiological studies, such as the incorporation of radioactive carbon dioxide into lignin as well as other plant constituents.

The organic chemists have expended a great amount of ingenuity, energy, and time in synthesizing coniferyl alcohol with the radioactive carbon atoms in known positions (23, 24). Parallel to the synthesis of the starting material has been the elaboration of quantitative degradation procedures to recover the radioactivity from lignin, more particularly from the vanillin (25) obtained by the oxidation of lignin, or from the "Hibbert Ketones," obtained by ethanolysis (26). The radioactive compounds have been administered to the plant in various ways: solid substances have been implanted under the bark and solutions have been absorbed through the ends of freshly cut stems. The localization of the introduced materials has been followed by radioautography. The proof that the radioactivity actually was incorporated into lignin rests upon the assumption that pre-extraction of the tissue with solvents such as alcohol, benzene and water, in which the synthetic materials are soluble under laboratory conditions, removed all material not converted to lignin. In some instances, isolated lignins were prepared by conventional methods prior to degradative recovery of radioactivity. The resulting data

have been regarded as proof that coniferyl alcohol participated directly in lignin synthesis (27). Analogous studies have been made with sinapyl alcohol and *p*-hydroxy cinnamyl alcohol (*loc. cit.*).

The second group of experiments attempted to relate lignification to the postulated precursors of coniferyl alcohol. Shikimic acid (28), phenylpyruvic acid, phenylalanine, tyrosine, ferulic acid, pyrocatechuic acid, vanillin, and other compounds less closely related to coniferyl alcohol have been "fed" to plants (29). Obviously, the amino acids and their precursors can enter various pathways, some of which do not terminate in lignification. Hence, it has been assumed that the greater the radioactivity recovered in the lignin or its degradation products the more direct was the relationship between the introduced chemical and the process of lignification. The data have been interpreted as supporting the current speculation about pathways to lignification.

Radioactive carbon dioxide introduced by the photosynthetic process has been used to follow the incorporation of carbon into lignin, cellulose, and other plant constituents (30, 31). This line of investigation has not received as much attention as the first two.

In addition to indicating the sources of skeletal carbon of lignin, C^{14} has been used to reveal precursors of the methoxyl groups. In barley and tobacco there was "a direct transfer of methyl groups" from the sulfur in methionine to oxygen in lignin. Formic acid was also transformed into lignin methoxyl, but apparently much less readily (32).

Enzymes

Complementary to the chemical interest in precursors is the biological question of what enzymes convert coniferin, etc., into lignin. So far there have been few direct observations. An active glucosidase, localized in the cell wall, has been detected in the developing wood cells of spruce prior to lignification. Microchemical observations with *P. radiata* and *E. goniocalyx* have indicated that a peroxidase generally was present in the cytoplasm and in the cell walls of those regions where lignin was absent. That is, the peroxidase distribution was complementary to the occurrence of lignin. Within the cells, peroxidase activity disappeared first from the cell corners and then centripetally through the cell wall as lignification progressed. In conifers the amount of peroxidase in the cambial zone was said to have an inverse relation to the amount of coniferin produced (33). Extensive model experiments with synthetic coniferin and coniferyl alcohol, together with related organic compounds, have shown that redoxases (partly purified mushroom oxidases) transform coniferyl alcohol into a series of intermediate compounds and ultimately into a ligninlike "dehydrogenation polymer" (DHP). The same intermediates have been isolated from spruce. Of the three intermediate dimeric compounds, the guaiacylglycerol-beta-coniferyl-ether was regarded as the more important for subsequent polymerization into lignin, but each of the three was capable of undergoing polymerization by the enzymes. The conditions of enzyme action were altered to favor the accumulation either of

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intermediates or of polymers. Sinapyl alcohol was copolymerized with coniferyl alcohol by the redoxases to a product which resembled hardwood lignin, but was not polymerized by itself (34). The enzyme in mushroom oxidase which produced DHP from coniferyl alcohol was not a polyphenoloxidase (35).

Other attempts to parallel lignin formation have employed eugenol as the principal substrate with peroxidases and hydrogen peroxide. By itself this system did not produce ligninlike polymers. It did do so, however, in tissue slices, in the presence of cell wall fragments, of cellulose and some other colloids, even inorganic materials. This led to the suggestion that a suitable "matrix" is a necessary part in the process of lignification. Surprisingly, methylcellulose was more active than cellulose in the form of filter paper. Moreover, this system converted thymol into a ligninlike material. Thymol belongs to the terpene (C_{10}) series and differs structurally from the C_6-C_3 skeleton which is generally regarded as characteristic of lignin precursors (36).

Tissue Culture

Some of the foregoing research on lignification has bordered on typical tissue culture. Attempts have been made to induce lignification under controlled conditions in tissues derived from both tree- and nontree-forming species. Calluslike tissue derived from secondary cambium of willow, hawthorne, and jack pine lost its ability to differentiate on successive transfers. In particular, tracheids became progressively fewer. However, callus from redwood burl continued to form tracheids after numerous transfers and spontaneously regenerated primary meristems leading to bud and shoot formation. Elm, birch, and basswood showed various degrees of differentiation. Although calluses formed by such cultures have exhibited lignification in the anatomical sense, no evidence has been adduced to show that there was lignification in the chemical meaning of the term.

The net result pointed "to distinct differences in the functioning of the endogenous mechanism which controls growth and differentiation . . . " (37). Also "There would seem to be good prospects that endogenous lignin formation may be controlled by specific substrates to a much higher degree in such cultures than in whole plants or in nongrowing excised parts" (37).

LIGNIN ISOLATION

One of the unresolved obstacles to the study of lignin is the lack of an assured method of isolating lignin in its natural state. During the period under review, advances have been reported in both biological and mechanical methods bearing on this problem. Conversely, the observations resulting from efforts at isolation have been used to support concepts of the form which lignin possesses in the plant.

Fungi

Because of the supposed specificity of enzyme action an extended effort has been made to isolate lignin, presumably unchanged, from wood and other lignified tissue through the action of fungi. There is no doubt that the brown rots break down the cell wall and that the yields of extractable or "liberated lignin" have been increased to as much as 23 per cent of the lignin originally present. The similarity in physical and chemical properties of the "liberated lignins" and the "Brauns' native lignins" has been taken as proof that the former actually existed as such in the original tissue. However, this procedure has the serious drawback that 13 to 15 months have been required to achieve the indicated yields (8, 38).

A unique instance of the biological preparation of lignin is the collection of the wood residues left by the West Indian termite. This insect digests the carbohydrates and excretes the lignin (39).

Ultrafine Wood

The ultrafine grinding of wood in a vibratory ball mill increases the solubilities of the wood and of its separate components considerably, which is undoubtedly a help (40). However, grinding cellulose in a vibratory ball mill caused some depolymerization and this raises the question of whether the greater solubility of lignin is due in part to a lower molecular weight and not only to greater accessibility to the solvents.

That the grinding affected the wood was evidenced by ash analyses and by changes in other properties. Whereas the flour was nearly white at the end of 5 hr. of grinding, it became somewat gray with 48 hr. grinding. Moreover, "typical values for ash content were 0.20 per cent for the wood and 0.28, 0.50, and 1.4 per cent for the 1-, 5-, and 48-hour products, respectively." It was further observed that "the material ground for 48 hours is more soluble (than after 5 hours grinding); 1.0 *N* alkali dissolves it, and even 0.1 *N* alkali dissolves a considerable portion. The powder produced in the 5-hour grind slowly swells and dissolves in 85 per cent formic acid. . . . It will dissolve in cold (−20°C.) conc. hydrochloric acid . . . with well purified aqueous lithium bromide, ultraviolet spectra can be obtained of the whole wood (48-hr. grinding?) in solution" (41).

Of particular interest to biologists is the attempt to isolate lignin from ultrafine wood by means of enzymes acting on the cellulose and other higher carbohydrates. The reaction was very much more rapid and complete than that of brown rot organisms on sawdust as the following description indicates.

> A commercial enzyme, Rohm and Haas, no. 19, rapidly digested the bulk of the carbohydrate in powdered wood. Normally, 1 gram of the powder was treated with a mixture containing 10 ml. of enzyme solution (10 per cent enzyme solids, after filtration), 2 ml. of 0.5 *M* acetate buffer with pH of 4.6, and enough water to give a total volume of 20 ml. . . . With a grinding time of 5 hr., about 95 per cent of the carbo-

hydrate is dissolved in 4 to 6 days, leaving 30 to 32 per cent of a lignin residue that contains 12 to 14 per cent residual carbohydrate. Only a small portion of the lignin passes into the enzyme solution. (*loc. cit.*)

One appraisal of ultrafine grinding was:

The subdivision of a material by milling is a rather rough method of obtaining a better knowledge of its structure. . . . The use of the results as a basis for conclusions about the physico-chemical structure of native wood is of doubtful value. However, because of the difficulties inherent in alternate methods, the products obtained are useful for chemical studies of the structure of lignin and lignin-carbohydrate complexes (42).

Another judgment read:

Indications are that the protolignin of wood is by nature, regardless of its association with the carbohydrate components, insoluble in solvents that do not react with it chemically. Since it becomes soluble when finely ground, this process must alter the lignin to some extent. However, this change is probably less drastic than that caused by isolation processes involving the use of strong acids, alkalies, or high temperatures (41).

Lignin-Carbohydrate Bond

It has long been recognized that the presence of lignin in woody tissues modifies the properties of cellulose, e.g., the cellulose is not readily dissolved by cuprammonium hydroxide. The question of whether this is caused by a mechanical incrustation of the cellulose by lignin or by a chemical combination between the two has been discussed pro and con for a long time. The great increase in the manufacture of paper and of rayon from wood has given economic importance to the theoretical question. This has been evident in the recent discussions of the existence of a "lignin-carbohydrate bond."

An extensive review concluded:

It can now be accepted without question that definite complexes of lignin and carbohydrate have been isolated. By heating wood with water, and by chlorination, ethanolysis, acetylation, and pulping (chlorite, sulfite, and kraft), compounds have been isolated which contain no free carbohydrates, but which liberate lignin and sugars on hydrolysis. In particular, the work of Traynard and of Kawamura and Higuchi has provided more than adequate evidence on this point. Since such complexes have been isolated under widely different conditions, it can be accepted that the linkage was present in the wood, rather than formed during isolation, and it can be accepted that at least part of the wood exists as a lignin-carbohydrate complex (9).

But a subsequent judgment stated:

In spite of the information gained, the type of association between the lignin and carbohydrate in wood remains speculative. It appears evident, however, that there is some kind of attachment—whether this attachment is by acetal or ether linkages, by secondary forces such as hydrogen bonds, or by a sort of mechanical entanglement, is not yet clear. It does seem unlikely, however, that the attachment involves a specific kind of sugar in the carbohydrate chain, since the lignin complexes contain all five sugar types (41).

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PHYSICAL PROPERTIES

The physical properties of lignin continue to be investigated from many different viewpoints. A cursory listing of some recent work may be helpful to the physiologist or plant chemist in selecting physical methods to aid his researches.

X-ray diffraction measurements and electron microscopy have given direct evidence that lignin is largely deposited between the microfibrils of cellulose and have indicated a slight association with the paracrystalline cellulose (17). The accessibility of lignin to solvents and to chemical reagents, including enzymes, has been greatly increased by ultrafine grinding. The ability of solvents to dissolve or swell a variety of isolated lignins increased as their hydrogen bonding capacity increased and as their solubility parameters (Hildebrand) approached a value of about 11 (43). Brauns' native lignin from black spruce, fractionally precipitated from dioxane with benzene, exhibited a molecular weight range from 2800 to 6700 (osmotic) or 2800 to 4800 (viscosity). Also there were differences in the infrared spectra of these fractions, particularly at those wavelengths which correspond to the carbonyl group (44). Other data have indicated that "liberated" and "native" lignins from a given wood sample (Scots pine, oak, birch, maple) are identical in the infrared region (45). Extensive comparisons have been made between the ultraviolet characteristics of model substances and a variety of lignins or lignin derivatives. Changes in absorption brought about by changes in pH ($\Delta\epsilon$ method) have been related to the phenolic groups in lignin preparations, e.g., in establishing the similarity between "native" lignins and Freudenberg's dehydrogenation polymer (DHP) (46). On the basis of x-ray studies (47), as well as other properties, lignin is generally considered to be amorphous. Chromatography has been used increasingly in the separation of lignin fractions (48). Separations have also been attempted by electrophoresis (45, 49). The resolution of "native" lignin has been achieved with countercurrent extraction (Craig) techniques [Freudenberg (50)].

MICROBIOLOGY

The massive literature on the relations between microorganisms and lignin has been the subject of two recent reviews (10, 11). Apparently it is not possible to generalize from the empirical facts. There is little doubt that some organisms attack primarily the nonlignin portions of plant structures, whereas others attack the lignin first, witness the "brown rots" and the "white rots" of wood. For specific references on such topics as the fate of lignin in digestive metabolism, its decomposition in soil and water, the decomposition of wood or wood wastes, and the formation of humic acids or coal, the reader should consult the above reviews. Real progress in many of these areas appears to depend upon a better definition of the nutritional or other metabolic requirements of specific microorganisms and upon better means of isolating and purifying the enzymes which cause the observed microbiological effects.

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REVIEWER'S COMMENTS

The chemical study of lignin is dominated by the premise that spruce lignin is a polymer of coniferyl alcohol. Most chemical work also proceeds from the further premise that spruce lignin is a prototype for other lignins even though they differ in their properties and in their chemical composition. At the same time, the idea has been widely accepted that chemically isolated lignins have been altered. One consequence has been to focus more attention again on lignin as it occurs in wood.

An important attempt to isolate unaltered lignins has been the use of "brown rots" to remove the polysaccharides. The similarity between the lignins so "liberated" and Brauns' "native" lignins has been regarded as evidence that little change occurred in the lignin structure. Unfortunately, the action of the living organisms may not be specific and the incubation period has been excessively long.

Another important development has been the ultrafine grinding of wood. It too has its limitations, but in combination with enzymatic decomposition of the carbohydrates, should lead to greater progress.

The most important development, however, is the simultaneous consideration of the biological, chemical, and physical aspects of lignin. Freudenberg, who claims that the essentials of lignin structure have been revealed (51), has asserted that biogenetic considerations provided the clues which were needed to enlarge the previous techniques of polymer chemistry to fit the lignin problem.

As soon as one tries to think comprehensively about the botanical setting of the lignin problem, many more questions arise than have been answered. If coniferyl alcohol is the essential monomer of lignin, why has it not been detected more readily in all woody plants? Is the accumulation of coniferin an integral part of lignification, or merely an incidental offshoot of a more general biochemical mechanism? If lignification starts in the outer wall and near the intercellular spaces, are the postulated enzymes actually present in these loci? In other words, is the cytoplasm still functioning within the thickened cell walls and in the middle lamella? Or is the deposition extracellular and perhaps in the nature of the coagulation of a colloidal emulsion (52)? If there is a lignin-carbohydrate bond, as alleged, what is the nature of the enzyme that can chemically unite a polymeric carbohydrate, which is already part of a cell wall, and a structural unit of the lignin eventually formed? Or if we prefer to regard lignification as a physical process, are the cell wall carbohydrates merely a "matrix" for the precipitated or coagulated lignin? And finally, for any given question do we have to consider only the one or the other extreme alternative?

In short, it would seem that the renewed biological interest in lignin, coupled with chemical and physical considerations, promises a lively and fascinating future for research in this field.

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FAT METABOLISM IN HIGHER PLANTS[1,2]

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During the past ten years the field of lipide biochemistry in higher plants has expanded very rapidly. This increased rate of progress can be traced to (*a*) the development of new techniques, such as reversed phase paper chromatography, gas-liquid chromatography and column chromatography, which are suitable for the analysis of small amounts of lipoidal material, (*b*) the use of isotopically labeled substrates, especially in studies with isolated enzyme systems which are very sensitive to substrate concentrations, and (*c*) the increased knowledge of lipide biochemistry in the animal field. Past volumes of the *Annual Review of Biochemistry* have thoroughly examined the literature pertaining to lipide biochemistry particularly in animal tissues. The reader is also referred to the reviews by Lynen (1) and Green (2). Although much work has been done in analyzing a vast array of fats and oils from many sources, the reviewers have, however, limited themselves to the dynamic aspects of the subject in higher plants. The third edition of Hilditch's *Chemical Constitution of Natural Fats* (3) continues to serve as a considerable source of information. In addition an important and highly valued collection of articles on many aspects of lipide biochemistry of plants is to be found in the *Handbuch der Pflanzenphysiologie*, volume VII (4).

Space limitations have not permitted the present reviewers to discuss the extensive literature concerning the field of lipoxidases. However, several complete reviews are available (5, 6, 7) and these should be consulted for information.

LIPIDE BREAKDOWN

Hydrolysis—Lipases

In recent years a considerable amount of work has been done on the enzyme lipase from a wide variety of plant tissues.

Singer & Hofstee (8, 9) showed that a partially purified, soluble lipase from wheat germ was able to hydrolyze a large series of water-soluble simple esters and mono- and tri-glycerides, as well as Tween 20 and 40. This wheat

[1] The survey of literature pertaining to this review was concluded October, 1958.

[2] The following abbreviations are used: AMP (adenosine monophosphate); ATP (adenosine triphosphate); CoA (coenzyme A); DNP (dinitrophenol); DPN (diphosphopyridine nucleotide); DPNH (diphosphopyridine nucleotide, reduced form); FAD (flavin-adenine-dinucleotide); GSH (reduced glutathione); PP (pyrophosphate); RQ (respiratory quotient); TCA (tricarboxylic acid); TPN (triphosphopyridine nucleotide); TPNH (triphosphopyridine nucleotide, reduced form).

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germ lipase was inhibited by sulfhydryl reagents. However, the facts (*a*) that complete inhibition toward one substrate could be demonstrated with little effect toward other substrates at the same inhibitor concentration, and (*b*) that the substrates with the smallest Michaelis constants (i.e., the substrates with the greatest affinity for the enzyme) allowed the greatest inhibition by —SH reagents, provided evidence that the —SH groups of this lipase are not directly involved in its activity. Singer (10) suggested that the —SH groups of the lipase might be located close enough to the active center so that the formation of complexes or the oxidation of these —SH groups might sterically interfere with the approach of the substrate molecules. This could account for the influence of the molecular dimensions of the substrate on the inhibition observed. From studies on the hydrolysis of substituted phenyl acetates by wheat germ lipase, Gawron *et al.* (11) also concluded that the —SH groups of this enzyme do not participate in the hydrolytic reaction.

Martin & Peers (12) showed that an oatmeal lipase, which was purified some 2000-fold, had a pH optimum of 7.4, a temperature optimum of 37 to 38°C. and a Michaelis-Menten constant of 0.006 *M* when tributyrin was the substrate. This purified lipase split off one butyric acid radical only from tributyrin and did not hydrolyze the various mono- and di-butyrins at pH 7.4 and 37°C.

Studies on castor bean lipase have been numerous. Ramakrishnan and his co-workers have investigated the conditions for maximum synthesis and hydrolysis of a variety of esters (13 to 20).

Bamann *et al.* (21) showed that the lipase extracted from unripe seeds or vegetative organs of *Ricinus* had a pH optimum of 8.5 to 10.5, whereas that extracted from ripe ungerminated seeds had a pH optimum of 4.7. Similarly, Yamada (22) observed that the lipase (spermatolipase) from the endosperm of ungerminated castor beans had a pH optimum of 4.5 to 5.0. However, during germination another lipase (blastolipase) with a pH optimum of 6.8 appeared first in the decotylated embryo and later in the endosperm.

Nizamuddin & Kulkarni (23) have presented evidence that castor bean lipase selectively splits off unsaturated fatty acids from peanut, safflower and linseed oils.

Some observations on the optical specificity of seed lipases have been reported by Bamann *et al.* (24). In experiments with mandelic acid esters the lipases of *Nigella damascena* and *Chelidonium majus* preferentially split the (+) ester, while those of *Nigella sativa* and *Ricinus communis* split the (−) ester of the racemate. These preferences were not absolute, however, and varied depending upon the method of extraction of the enzyme and upon the concentration of the substrate.

The changes in the activity of lipase during the maturation of castor bean seeds (25) and during the germination of peanut (26), rapeseed (27) and castor bean seedlings (28) have been reported.

Some of the properties of lipases from a wide variety of seeds including *Sesamum indicum, Arachis hypogea, Guizotia abyssinica, Carthamus, Brassica*

nigra, *Gossypium herbaceum* (29), lettuce (30, 31), rye (32) and oil palm (33) have also been described.

In addition, lipase has been detected on the surface layers of the root tips of some higher plants (34) and in a wide variety of plant tissues including the basswood of a number of trees (35).

Intact Tissues

The consumption of storage reserves of fat by germinating seedlings has been known for well over 100 years. However, only recently has the development of new techniques permitted the convenient quantitative determination of changes in the individual fatty acid components. A column chromatographic procedure for the determination of milligram quantities of a wide range of both saturated and unsaturated fatty acids has been developed by Crombie *et al.* (36), who applied this method to the investigation of fat metabolism in *Citrullus vulgaris* and *Elaeis guineensis*.

The storage fat in *Citrullus* cotyledons is highly unsaturated and contains approximately 70 per cent linoleic, 7 per cent oleic, 10 per cent palmitic and 11 per cent stearic acids together with smaller amounts of arachidic and octadecatrienoic acids. Crombie and her co-workers studied the consumption of this fat during the germination of *Citrullus* seedlings in both the light (37) and the dark (38). In the light the fat was consumed most rapidly when the cotyledons expanded and turned green and the major fatty acids in the oil were broken down at rates proportionate to the quantities originally present, except for oleic acid which was metabolized somewhat more rapidly. There was no evidence for the accumulation of free fatty acids or of short chain fatty acids during this rapid consumption of fat. Since these workers showed that estimates of free fatty acids obtained by titration of the oil were unreliable, it would appear that many of the older reports of the occurrence of free fatty acids require re-examination. After the consumption of the storage fat, small quantities of a typical leaf fat, in which linolenic acid comprised about 60 per cent, were formed in the *Citrullus* cotyledons as the photosynthetic function developed. Fat consumption occurred in essentially the same way when these seedlings were germinated in the dark. Again, there was no accumulation of free fatty acids. However, the protoplasmic fat left in the cotyledons after the disappearance of the storage fat contained a smaller proportion of linolenic acid than that from green cotyledons of a corresponding age. Both in light- and dark-grown seedlings the weight of phosphatide per cotyledon increased until the eighth day and thereafter declined in the dark but continued to rise in the light.

The non-accumulation of free fatty acids during the consumption of a cotyledonary fat has also been reported from experiments with etiolated sunflower seedlings (39).

The consumption of endospermic fat in seedlings of the West African oil palm (*Elaeis guineensis*) which were germinated in the light was followed by Boatman & Crombie (40). This storage fat consists of an unusual mixture

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of fatty acids, the most abundant of which are saturated C_{10}–C_{16} acids. During germination the endosperm is invaded by the haustorium which is an absorptive organ formed by the developing embryo. Free fatty acids accumulated in the endosperm and were absorbed by the haustorium without prior conversion to carbohydrate. However, no free fatty acids were detected in the haustorium itself. These results have been interpreted in terms of re-esterification of the free fatty acids during the absorption process. The fats of the endosperm and haustorium are of similar composition, but the quantity which accumulates in the haustorium can only account for a fraction of the endospermic fat which is consumed. The fate of the remainder is somewhat obscured by simultaneous photosynthesis, although, before the start of photosynthesis, respiration can account for much of the decrease in the fat content.

The consumption of the stored endospermic fat in castor bean seedlings is quite different from that described for the oil palm. Although free fatty acids apparently accumulate also in the castor bean endosperm (41), the lipides are converted into carbohydrate before translocation (42, 43).

The conversion of the products of fat degradation into carbohydrate and other substances will be discussed later.

β-Oxidation

The β-oxidative fatty acid cycle, which was elucidated from experiments with animal tissues, is illustrated in Fig. 1.

Early evidence for β-oxidation in higher plants was obtained from experiments with intact plants. In 1939 Grace (44) observed that the growth-promoting activity of ω-substituted aryl or aryl-oxyalkyl carboxylic acids was correlated with side-chain length, since an alternation of activity occurred as the homologous series was ascended. When the first five members of the ω-(1-naphthyl)alkylcarboxylic acid series were examined for the capacity to promote rooting of cuttings, only those compounds with an odd number of methylene groups in the side chain possessed activity similar to that of 1-naphthyl-acetic acid; the compounds with an even number of methylene groups had very low activity. In 1947, Synerholm & Zimmerman noted (45) that a similar alternation of activity occurred with increasing length of side chain in the series of ω-(2,4-dichlorophenoxy)alkylcarboxylic acids and suggested that this alternation of activity could be explained in terms of β-oxidation of the side chains.

Fawcett *et al.* in 1954 (46) supplied members of the homologous series of ω-phenoxy acids $C_6H_5O(CH_2)_nCOOH$ to flax seedlings and after a suitable time period asayed the tissue for phenol. Appreciable quantities of phenol were produced only from those members which possessed an even number of side chain methylene groups. These results support the concept of side chain degradation by a β-oxidation mechanism. Similar results have been obtained by Luckwill & Woodcock (47), who showed that ω-(2-naphthoxy)alkylcarboxylic acids could induce parthenocarpic development in tomato ovaries.

Wain & Wightman (48) demonstrated in 1954 that ring substituted ω-phenoxyalkylcarboxylic acids such as 4-chloro, 2,4-dichloro, 2-methyl-4-chloro derivatives show a typical alternation of biological activity when analyzed either by the wheat cylinder test or by the pea curvature and tomato epinasty tests. However, while the 2,4,5-trichlorophenoxyalkylcarboxylic acids with an odd number of side chain methylene groups were active in the wheat cylinder test, only 2,4,5-trichlorophenoxyacetic acid had biological activity in tomato and pea tissue. Wain & Wightman concluded that

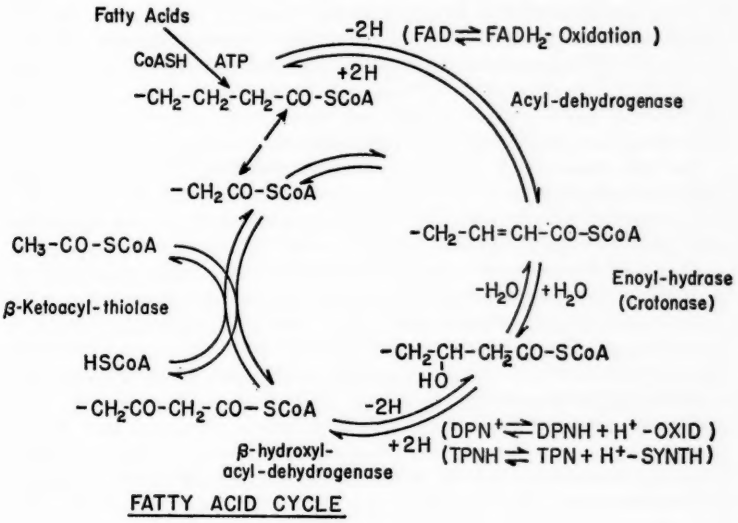

FIG. 1

while enzymes participating in β-oxidation have relatively broad specificities, their ability to degrade the side chain of a specific ω-phenoxyalkylcarboxylic acid depends both upon the source of the enzymes and upon the nature and position of the nuclear substituents of the substrate.

Since amides of 2,4-dichlorophenoxyalkylcarboxylic acids possess the same alternation of biological activity with a variety of tissues, these amides are probably first hydrolyzed to the corresponding acids which are then degraded to the acetic acid derivative or to dichlorophenol, depending upon the homologous series derivative employed in the tests (49).

Phenoxyalkyl nitriles appear to participate in a more indirect side chain degradation which involves a combination of α- and β-oxidations. This aspect will be covered more completely in the discussion to follow on α-oxidation.

Although much *in vivo* evidence suggested a β-oxidation mechanism in

plants, a cell-free plant system that would oxidize long chain fatty acids has only recently been described. In 1952 Link *et al.* (50) noticed that butyrate-1-C^{14} was metabolized to $C^{14}O_2$ by slices of tomato stems. In the following year Newcomb & Stumpf (51) incubated slices of germinated peanut cotyledons with radioactive substrates for six hours under aerobic conditions and detected considerable $C^{14}O_2$ in the respiratory CO_2. Thus the radioactivity in the respiratory CO_2 from acetate-1-C^{14} was 29 per cent, acetate-2-C^{14} 10.5 per cent, butyrate-1-C^{14} 20.5 per cent and hexanoate-1-C^{14} 42 per cent of the original activity. Smillie [1955 (52)] prepared particles from etiolated pea seedlings and noticed that butyrate, in the presence of phosphate, Mg^{++}, adenylic acid, and malic acid, gave a transient increase in oxygen uptake. Within 15 to 20 minutes, however, the initial rate fell to that of malate oxidation alone. In 1955, Poljakoff-Mayber (53) observed that the endogenous respiration of particles from germinated lettuce seedlings was sharply curtailed when long-chain fatty acids were tested as potential substrates.

In 1954 Millerd & Bonner (54) demonstrated that the enzyme which catalyzes the activation of acetate to acetyl CoA in the presence of ATP and CoA was present in spinach and wheat embryos and in some other plant tissues. Acetoacetic acid was also formed in small amounts in these systems.

Stumpf & Barber in 1956 (55) succeeded in demonstrating a β-oxidative degradation of a large number of fatty acids in a cell-free system. Essential for vigorous oxidation of fatty acid were the following conditions: (*a*) low concentrations of fatty acids, (*b*) freshly prepared peanut mitochondria, and (*c*) a surprisingly large number of cofactors. The specific requirements for maximum oxidation of fatty acids were: ATP, CoA, DPN, TPN, Mn^{++}, a TCA cycle acid, and GSH. Cytochrome-*c*, thiamin pyrophosphate, flavin monophosphate, and flavinadeninedinucleotide were not necessary. In contrast, requirements for CoA, DPN, and TPN by intact animal mitochondria have not been observed (1, 2). Recent work by Beevers & Walker (56) has also indicated that the oxidation of TCA cycle acids by mitochondria from 4-day-old *Ricinus* endosperms requires a rather extensive complement of cofactors including ATP, DPN, thiamine pyrophosphate, and CoA. It would seem either that these cofactors dissociate easily and are separated from the mitochondrial enzymes in the course of the preparation or that they are present in suboptimal amounts in the mitochondrion as it exists in its milieu.

Oxidation of all the straight chain aliphatic acids from acetate to stearate by the complete mitochondrial system was good with the exception of the structural isomers isobutyrate, isovalerate, and isocaproate. The odd-chain acids propionate and valerate were also oxidized vigorously.

The evidence for β-oxidation and TCA cycle participation by this isolated peanut system is summarized below. The components required for oxidation of fatty acids by the peanut mitochondria are, in general, those involved in β-oxidation by animal mitochondria and by the soluble multienzyme systems described by Green and Lynen. This similarity of component requirements suggests that the peanut mitochondrion can effect fatty

acid oxidation through the same or a similar group of reactions. Malonate, a competitive inhibitor of succinate oxidation and thus of the TCA cycle, inhibits the oxidation of butyrate about 75 per cent at a final concentration of 6×10^{-3} *M*. Since citrate is handled asymmetrically by the enzymes of the TCA cycle, at least three turns of the cycle are required before an even-numbered carbon atom of a fatty acid (the methyl group of acetyl CoA) appears in the respiratory CO_2. On the other hand, an odd-numbered carbon atom of a fatty acid (the carboxyl group of acetyl CoA) would be detected in the respiratory CO_2 as soon as the second turn of the cycle is completed. When palmitic acid labeled in either the second or the third carbon atom and carboxyl- and methyl-labeled acetate were compared as to rate of $C^{14}O_2$ release, the results supported the hypothesis that the peanut mitochondrial system oxidizes fatty acids through a β-oxidative pathway. The participation of the TCA cycle in the terminal oxidation of butyrate was indicated by the labeling of the TCA cycle intermediates when butyrate-1-C^{14} was oxidized by the complete system.

It was of interest to investigate acetoacetate accumulation in the peanut mitochondrial system during active butyrate oxidation since such accumulation is generally observed when fatty acids are oxidized by liver mitochondria. The accumulation of acetoacetate may be enhanced by blocking its utilization through the TCA cycle by such procedures as the addition of malonate or DNP or the omission of a TCA acid. When butyrate-1-C^{14} was oxidized for two hours by the complete peanut mitochondrial system, 11 per cent of the total C^{14} was released as respiratory $C^{14}O_2$ and 3 per cent appeared in acetoacetate. In the presence of malonate the respiratory $C^{14}O_2$ was depressed to 1 per cent while 2.4 per cent of the C^{14} was present in acetoacetate.

Stanley & Conn (57), in studying the range of activity of mitochondria from pine seedlings, have observed that $C^{14}O_2$ is released from palmitate-1-C^{14} and butyrate -1-C^{14}.

The logical sequel in the elucidation of the β-oxidative system in plants is the isolation and purification of the enzymes responsible for the individual reactions. While the activities of the fatty acid kinase, butyryl dehydrogenase, crotonase and thiolase have been observed, no concerted efforts involving the purification of the crude extracts have yet been undertaken.

α-Oxidation

In 1952 Newcomb & Stumpf (58) observed that two cell-free fractions obtained from cotyledons of germinated peanut seedlings released $C^{14}O_2$ from palmitate-1-C^{14}. The first fraction consisted of yellow submicroscopic particles which were sedimented by centrifuging at $100{,}000\times g$ and corresponded to microsomes isolated from animal tissues. Release of $C^{14}O_2$ from palmitate-1-C^{14} required no cofactors, whereas DPN was required for the release of radioactivity from palmitate-3-C^{14}. Neither Krebs cycle acids nor CoA and ATP were required. The second fraction was the supernatant solu-

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tion which remained after removal of both the microsomal and mitochondrial particles from the crude cotyledon homogenate. This fraction catalyzed the vigorous release of $C^{14}O_2$ from palmitate-1-C^{14} but had no activity towards palmitate-3-C^{14}. When the proteins from the supernatant fraction were precipiated by 80 per cent saturated ammonium sulfate and were redissolved in buffer, they had no activity unless a portion of boiled crude supernatant fraction was added. In 1952 the mechanism of oxidation by both fractions was quite obscure.

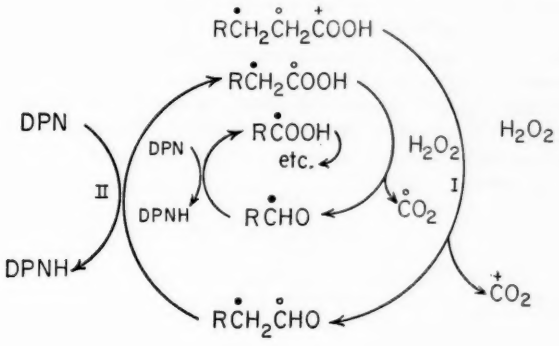
In 1954 Humphreys *et al.* (59) examined the microsomal system extensively and reported that palmitate-1-, -2-, -3-, and -11-C^{14} were oxidized but in decreasing order. Palmityl-1-C^{14} CoA was inactive. DPN was required for the release of C^{14} from internally labeled palmitate (-2-, -3-C^{14}, etc.) but not from palmitate-1-C^{14}. The C_{18}, C_{16}, C_{14} saturated acids were active substrates, but laurate (C_{12}) had negligible activity and the lower acids had none. In 1955 Humphreys & Stumpf (60) solubilized the enzyme system associated with the microsomes by exposure to sodium choleate. The cofactor requirements of this soluble system were the same as those of the original microsomal particles, but after treatment of the preparation with ammonium sulfate an additional unknown cofactor from boiled extracts of peanut cotyledons was required.

At the same time Castelfranco *et al.* (61), studying the supernatant enzyme system which had limited terminal oxidative activity, showed that the cofactor which was found originally in boiled peanut homogenates was a mixture of glycolic and lactic acids. In the absence of either L-α-hydroxy or glycolic acids the supernatant enzyme system had no activity. No other cofactor requirements were demonstrated. $C^{14}O_2$ was readily released from α-keto palmitate-1-C^{14} but not from α-hydroxypalmitate-1-C^{14}. The product of the reaction was unknown.

A step towards elucidation of the nature of the supernatant oxidative system was taken when two observations were made in 1956 by Stumpf (62). He showed that glycolic acid and L-α-hydroxyaliphatic acids served as substrates for glycolic oxidase, which was present in low concentration in the enzyme preparation. In the oxidation of L-α-hydroxy acids small amounts of H_2O_2 were formed and were then employed for the peroxidation of the saturated fatty acids. A variety of H_2O_2-generating systems served equally effectively as replacements for glycolic acid. In addition a direct correlation between the formation of long chain aldehydes and the specificity of the enzyme towards substrate chain length was reported. Since both azide and cyanide served as sensitive inhibitors of the reaction, it was suggested that the enzyme involved in the supernatant system was a fatty acid peroxidase. Imidazole strongly inhibited both the microsomal and the supernatant systems.

The dual function of the microsomal and the supernatant fractions was clarified recently by Martin & Stumpf (63). They showed that at least two enzymes are involved in the internal oxidation of long chain fatty acids.

The first is a fatty acid peroxidase which peroxidatively decarboxylates the acid to an aldehyde and CO_2. The aldehyde is then oxidized by a DPN-specific aldehyde dehydrogenase to an acid containing one less carbon than the initial acid substrate. The peroxidase is sensitive to imidazole and azide. Its activity is restricted to the $C_{18}-C_{14}$ acids with the C_{15} acid being the most active substrate. The aldehyde dehydrogenase is DPN-specific and is present in the microsomes but not in the supernatant fraction. These results can be depicted as an α-oxidation as shown in Figure 2.



α-OXIDATION OF LONG CHAIN FATTY ACIDS

I Long chain fatty acid peroxidase

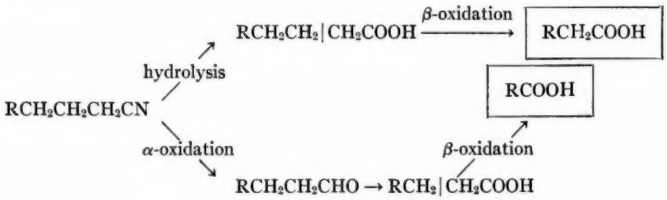
II Aldehyde dehydrogenase

FIG. 2

Another type of α-oxidation has been observed by Fawcett *et al.* (49, 64) who tested the growth regulating activity of a homologous series of 2,4-dichlorophenoxyalkyl nitriles by means of the wheat cylinder, pea curvature and tomato leaf epinasty tests. They showed that the wheat tissue catalyzed (*a*) the hydrolysis of the nitriles to the corresponding acids, and (*b*) the subsequent β-oxidation of the acids thus formed. In contrast, the pea and tomato tissues were able to hydrolyze only the acetonitrile and did not attack the higher members of the series. In the experiments with the wheat tissue, those members of the series which had an even-numbered side chain (i.e. the acetic, butyric and caproic derivatives) gave rise predominantly to 2,4-dichlorophenoxyacetic acid, whereas 2,4-dichlorophenol was the major product formed from the propionic, valeric and heptanoic nitriles. However,

the formation of some 2,4-dichlorophenoxyacetic acid from the valeric and heptanoic derivatives and the formation of some 2,4-dichlorophenol from the even-numbered members suggested the operation of some other degradation mechanism in addition to those of hydrolysis and β-oxidation. These results have been explained in terms of two distinct degradation pathways: (*a*) hydrolysis of the nitrile followed by β-oxidation, and (*b*) α-oxidation followed by β-oxidation of the α-methylene group.

To investigate this α-oxidation mechanism in more detail, Fawcett *et al.* (49, 64) examined the behavior of 3-indolylacetonitrile in wheat and pea tissues and showed that this substance was converted into 3-indolecarboxylic acid. 3-Indolealdehyde was identified as an intermediate in this conversion and was readily oxidized to the acid by both tissues. No trace of the aldehyde was detected, however, when 3-indolylacetic acid was incubated with these tissues. This fact together with other evidence suggested that 3-indolylacetic acid is not an intermediate in the formation of 3-indolealdehyde from 3-indolylacetonitrile. The two pathways of nitrile degradation in these tissues may be represented as follows:



Oxidation of Odd Chain Fatty Acids

Propionic acid is metabolized by various routes depending upon the type of organism concerned (65). Both plant and animal tissues can catalyze the conversion of propionic acid according to the following sequence:

$$\text{propionic acid} + \text{CoA} + \text{ATP} \rightarrow \text{propionyl CoA} + \text{AMP} + \text{PP} \qquad 1.$$

$$\text{propionyl CoA} \xrightarrow{-2\text{H}} \text{acrylyl CoA.} \qquad 2.$$

Acrylyl CoA can then undergo several different transformations as shown below.

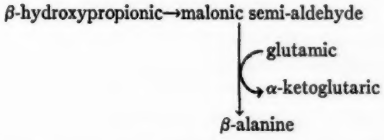
acrylyl CoA

- —*Clostridium propionicum* (66, 67) → β-alanyl CoA + NH_3 — 3a.
- —*Pseudomonas* (65), pigeon heart muscle, + H_2O → lactyl CoA — 3b.
- —crotonase from *C. kluyveri*, animal tissue, plant mitochondria (68, 72) → β-hydroxypropionyl CoA — 3c.

Acrylyl CoA aminase (3a) has been found only in *C. propionicum* (66, 67) and there is no evidence for its occurrence in animal or higher plant

tissues. The formation of lactyl CoA (3b) by an α-hydrase (65) is surprising in that according to organic chemical mechanisms the addition of H_2O should yield only β-hydroxypropionyl CoA. Both lactyl CoA and β-hydroxypropionyl CoA are inert in the *C. propionicum* system (66, 67).

In animal tissues (68), β-hydroxypropionic acid can undergo the following reactions:



The principal path of propionic acid oxidation in animal tissues, however, has been elucidated by Flavin, Ochoa and co-workers (69, 70, 71) as follows:

$$\text{propionic} \xrightarrow[\text{CoA, } Mg^{++}]{\text{ATP}} \text{propionyl CoA} \xrightarrow[\text{ATP}]{CO_2} \text{methylmalonyl CoA} \rightarrow \text{succinyl CoA}$$

There is no evidence that this sequence occurs in plant tissues.

In 1956 Stumpf & Barber (55) observed that peanut mitochondria, when supplemented with Mn^{++}, a Krebs cycle acid, ATP, and CoA, rapidly released $C^{14}O_2$ from either propionate-1-C^{14} or valerate-1-C^{14}. Giovanelli & Stumpf (72) re-examined this result and showed that propionic acid underwent reactions 1, 2 and 3c. β-Hydroxypropionic acid was isolated as an intermediate, was characterized, and was employed as the initial substrate for further studies. It could be shown that (*a*) propionate-1-C^{14} (or β-hydroxypropionate-1-C^{14}) released all of its C^{14} as $C^{14}O_2$ and other reaction products were completely lacking in C^{14}, (*b*) propionate-2-C^{14} (or β-hydroxypropionate-2-C^{14}) and propionate-3-C^{14} yielded $C^{14}O_2$ only after the Krebs cycle acids became labeled, but the release of $C^{14}O_2$ from carbon-2 was more rapid than from carbon-3. Acetyl CoA was neither isolated nor trapped, but succinate-C^{14} was obtained after the oxidation of either propionate-2-C^{14} or propionate-3-C^{14}. With propionate-2-C^{14}, the C^{14} was located exclusively in the methylene groups of succinate, whereas, with propionate-3-C^{14}, the C^{14} was confined to the carboxyl groups of succinate. The combined evidence suggested that (*a*) the carboxyl group of propionate is released exclusively as CO_2, (*b*) the methylene group becomes the CH_3 group of acetate, and (*c*) the methyl group of propionate is converted into the carboxyl group of acetate. The enzyme complex has also been found in mitochondria obtained from lupine cotyledons and avocado mesocarp. The sequence can be depicted by the following steps:

propionic → propionyl CoA → acrylyl CoA → β-hydroxypropionyl CoA →

β-hydroxypropionic → malonic semialdehyde → acetyl CoA + CO_2 →

TCA cycle complex → CO_2.

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At present the significance of this pathway in plant materials is not clear since odd chain fatty acids have not been found. It is conceivable, however, that propionic acid, produced during amino acid degradation, could be rapidly removed by this route to prevent toxic effects. Presumably valeric acid and other odd chain fatty acids are first degraded by β-oxidation to a residual C_3 acid, which is then oxidized by this pathway.

Glycerol Metabolism

The path of glycerol metabolism in plants appears to be similar to that observed in animal tissues. Thus the sequence glycerol→α-glycerol phosphate→triose phosphate→pyruvic acid has been substantiated in isolated enzyme systems obtained from homogenates of cotyledons of germinated peanut seedlings [Stumpf (73)]. When radioactive glycerol was added to a reaction mixture containing peanut mitochondria, a soluble peanut cytoplasmic protein fraction, and a number of cofactors and co-substrates, a rapid formation of $C^{14}O_2$ occurred. The omission of any member of the complete system resulted in diminished or complete loss of activity. Thus, absence of ATP, α-ketoglutarate or thiamin pyrophosphate seriously impaired the oxidative activity. Without Mg^{++} and DPN there was a decrease in activity of about 50 and 60 per cent respectively and the presence of either malonate or fluoride reduced oxidation to a very low level. These observations can therefore be interpreted as follows: (*a*) the necessary enzymes for the process which releases CO_2 are to be found in both the mitochondrial and the soluble protein fractions, (*b*) ATP presumably serves as a source of $\sim$P for the phosphorylation of glycerol and for the maintenance of the TCA cycle, (*c*) the requirement for a TCA cycle acid suggests the participation of the TCA cycle in the formation of $C^{14}O_2$, (*d*) the inhibition by malonate is related to the known capacity of malonate to inhibit succinic dehydrogenase, and (*e*) the inhibition by fluoride suggests the participation of enolase, which is the site of fluoride inhibition in the glycolytic sequence.

Glycerol phosphate dehydrogenase was located in the mitochondrion and the soluble protein had no dehydrogenase activity. No cofactors appeared to be required by the dehydrogenase and thus the rate of oxidation was influenced by neither DPN nor TPN. Cytochrome-*c* was, however, rapidly reduced. Since added cytochrome-*c* was not required for maximum dehydrogenase activity, it probably occurs in the mitochondria in a concentration that is not limiting. If cytochrome-*c* is indeed the redox acceptor for the system, then the plant α-glycerol phosphate dehydrogenase is similar to the particulate system found in animal tissues (74). The stoichiometry of the reaction suggests the following:

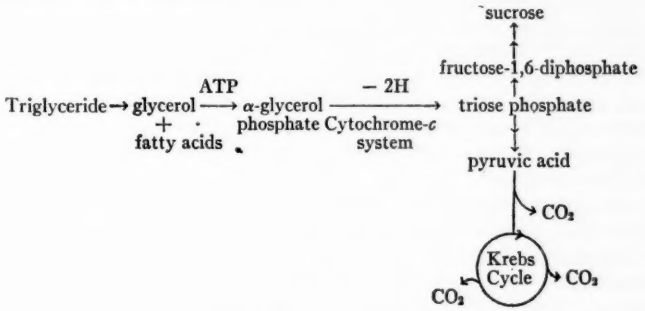
$$\alpha\text{-glycerol phosphate} + O \rightarrow \text{triose phosphate} + H_2O$$

When glycerol-1,3-C^{14} was oxidized by the complete reaction system in the presence of 10 μmoles of citrate as a sparker, appreciable radioactivity was found in several of the TCA cycle acids, particularly in citrate, malate, fumarate, succinate, and α-ketoglutarate in decreasing order.

Further data concerning the metabolism of glycerol was obtained by Beevers (75) when he studied the transfer of C^{14} from glycerol-C^{14} to other compounds in intact nongreen cotyledons from germinated castor beans. He showed that while part of the glycerol was degraded to $C^{14}O_2$, most of the C^{14} appeared in sucrose. Aerobic conditions were required for glycerol oxidation, suggesting the participation of a cytochrome-linked enzyme. Both fluoride and malonate depressed endogenous oxygen uptake and $C^{14}O_2$ release.

After a 6 to 7 hr. experiment in which one gram of castor bean cotyledons was incubated with 20 μmoles of glycerol-1,3-C^{14}, sucrose was isolated and hydrolyzed. The label was equally distributed between the glucose and fructose moieties. Degradation of the glucose showed that 97 per cent of its C^{14} was fairly evenly distributed among carbons 1, 3, 4, and 6. Evidently the intact carbon skeleton of glycerol was incorporated into glucose without prior degradation.

On the basis of these two papers the sequence of metabolism of glycerol can be summarized as follows:



SYNTHESIS

In Vivo

It is fairly well established that the synthesis of fat in ripening fruits and seeds occurs *in situ* from fat precursors, most probably carbohydrates, which have been translocated from other parts of the plant (76 to 81).

Crombie & her co-workers have studied the formation of fat in both the maturing exocarp and kernel of the oil palm (*Elaeis guineensis*). Fat formation in the maturing kernel (76) was followed from 10 weeks after pollination to full maturity (20 weeks) during which period the fat content increased one hundredfold. The fat in the mature kernel contained a mixture of seven different saturated fatty acids together with the unsaturated acids oleic (15 to 17 per cent) and linoleic (0.7 to 3.1 per cent). The major component of this highly saturated fat was lauric acid (46 to 49 per cent). At the earliest stages of ripening these acids were all present, but in very different proportions in which the unsaturated acids formed a much larger fraction. The re-

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sults indicated that the young kernels contained a small quantity of a largely unsaturated protoplasmic fat and that at a certain stage some physiological change in the tissue resulted in the formation of large quantities of a highly saturated storage fat. No fatty acid interconversions were demonstrated. The evidence also indicated that free fatty acids were not accumulated prior to esterification.

In contrast to the kernel fat, the major components of the oil which was laid down in the maturing exocarp of the oil palm were palmitic (41 to 45 per cent) and oleic (34 to 39 per cent) acids with smaller amounts of stearic and linoleic acids (77). The accumulation of fat in the exocarp was delayed until the kernel had almost finished developing and was then extremely rapid, most of the lipide being formed in one week. Again no evidence was obtained for fatty acid interconversions at any stage.

Many aspects of the biosynthesis of the unsaturated C_{18} fatty acids still remain to be elucidated. Some of the physiological features of this biosynthesis were reviewed by Hilditch (82). The earlier workers concluded that the unsaturated fatty acids were formed by desaturation of the saturated or less unsaturated fatty acids. The evidence to support this hypothesis consisted of the increase in the degree of unsaturation of the oil during maturation (83). However, this increase in unsaturation can also be interpreted in terms of the synthesis of relatively more of the more highly unsaturated acids in the later stages of ripening (82). Recent work by Simmons & Quackenbush (84) on the formation of fatty acids by developing soybean seed gave no evidence for dehydrogenation of saturated fatty acids in the formation of the unsaturated fatty acids. Earlier, Painter (85) had observed an increase in the iodine value of flaxseed oil during its deposition and stated that although the results could be interpreted to indicate that the unsaturated fatty acids were formed by desaturation, it was equally probable that the unsaturated acids were formed independently. Similarly, Franzke (86) concluded that the changes in the chemical characteristics of sunflower seed oil during its formation do not justify the conclusion that the fatty acids are dehydrogenated.

It is known that when a given species of seed is grown in a colder climate it tends to produce a more unsaturated fat than when grown in a warmer locality (82). Barker & Hilditch (87) analyzed the fatty acid composition of sunflower seed oil which had been formed in different localities in Africa. They found that oils with over 70 per cent of linoleic acid were obtained from sunflowers grown in cool climates while some oils from sunflowers grown in hot climates contained only 20 per cent linoleic acid and over 60 per cent oleic acid. Seeds of sunflowers from these different localities in Africa were grown side by side in England and the composition of the oil of these English-grown seeds consisted of 65 to 75 per cent linoleic and 15 to 25 per cent oleic acids in all instances (88). Similar experiments have been performed by Grindley (89) with sunflower seeds grown in both the winter and the summer at Khartoum. He also observed that the increased temperature

during the ripening of the seed led to a very much reduced linoleic acid content in the oil.

Hilditch (82) considers that these observations are consistent with the view that in the synthesis of C_{18} unsaturated acids the order of production of the final long chain acids is via the more unsaturated towards the least unsaturated (oleic) acids. In a cold climate the rate of production of the seed fat is naturally slower than in a warmer region, and at the same time the equilibrium reached in regard to the relative proportion of the unsaturated acids may differ, in that the ultimate production of the less unsaturated acids may not have proceeded so far. Thus, in a seed fat containing oleic and linoleic acids, relatively more linoleic acid might be expected in a fat produced at a lower temperature.

It should also be noted that the variation in the fatty acid composition of oil formed at different temperatures is confined to the unsaturated acids. In comparison, the saturated components show almost negligible variation in amount. Hilditch (82) suggests that this is good evidence that the unsaturated fatty acids are synthesized by a mechanism which is different from that for the saturated acids. In addition, the rate of synthesis of unsaturated acids *in vivo* is evidently more affected by temperature than is the synthesis of the saturated acids.

In Vitro

In 1952 Newcomb & Stumpf (51) studied the synthesis of long chain fatty acids ***in vitro*** by incubating slices of cotyledons from both developing and germinating peanuts with radioactive substrates. The intact cells of peanut cotyledon slices actively metabolized a variety of potential precursors of fatty acids, but appreciable incorporation into long chain fatty acids was observed only with acetate-1- and -2-C^{14} and evenly-labeled glucose and fructose. Thus acetate-1- and -2-C^{14} contributed to the greatest extent (for example, 22 and 34 per cent respectively in cotyledons from developing peanuts), whereas glucose and fructose contributed about 6 per cent of their initial activity. Butyrate-1-C^{14}, caproate-1-C^{14}, and valerate-1-C^{14} furnished 1 to 3 per cent of the initial activity, while the remaining substrates contributed less than 1 per cent. Because of the low degree of incorporation by these substrates into the long chain fatty acids, it is quite probable that they do not enter as intact units but rather are first degraded to C_2 fragments which are then directed into many pathways of metabolism, one being fatty acid synthesis. The C_1 unit, formate, was only negligibly utilized for fatty acid formation, as were pyruvate-2-C^{14} and succinate-1, 4-C^{14}, despite the fact that they contributed greatly to respiratory CO_2. The observation that the C_1 unit, formate, which was rapidly converted to CO_2, was quite ineffective as a fatty acid precursor suggests that CO_2 fixation is a relatively unimportant source of carbon in fatty acid synthesis.

Since fats are rapidly degraded in the cotyledons of germinating seeds, the percentage incorporation of acetate into the fatty acid pool in slices of

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such cotyledons is surprisingly similar to that which occurs in the slices of developing cotyledons. This would imply that long chain fatty acids are in a dynamic equilibrium with their C_2 precursors and that while degradation may be the principal pathway, a not-too-neglibible proportion of C_2 fragments is redirected to the resynthesis of long chain fatty acids.

Gibble & Kurtz showed in 1956 (90) that developing flax fruits utilized acetate-1-C^{14} in the synthesis of long chain fatty acids. The fatty acids were separated and the distribution of radioactivity was determined. The greatest isotopic level was found in the odd-numbered carbon atoms, which is consistent with the formation of long chain saturated and unsaturated fatty acids by means of the multiple β-condensation of acetate.

Simmons & Quackenbush [1954 (91)] studied the synthesis of individual fatty acids by excised soybean stems supplied with sucrose-C^{14}. After two days on a sucrose-C^{14} medium, oleate had the highest specific (and also total) activity of the several fatty acids reported, while stearate, linoleate and linolenate were labeled in decreasing order. After two days the radioactive sucrose was replaced by cold sucrose and fatty acid synthesis was allowed to continue. At the two-day stage 56 per cent of the total radioactivity was in the oleate fraction and only 18 per cent in linoleate. After five days oleate had retained 38 per cent of the activity but now the linoleate contained 35 per cent of the total. Simmons & Quackenbush suggested that the oleate may be preferentially produced from sucrose and then converted to more unsaturated fatty acids or to saturated fatty acids.

Shafizadeh & Wolfrom (92) prepared labeled fat by supplying D-glucose-6-C^{14} to maturing cotton bolls. Zabin (93) injected labeled acetate into semiripe tomatoes and permitted ripening to proceed for 5 to 12 days. Methyl- and carboxyl-labeled acetate were equally utilized for fat formation. Experiments in which the stems of maturing flax plants were placed in solutions of C^{14}-labeled sucrose, acetate-1-C^{14}, pyruvate-2-C^{14} and glycine-1-C^{14} were performed by Vyval'ko *et al.* (94), who showed that C^{14} from each of these substances was incorporated into fat in the ripening seeds.

In 1957, Kurtz & Miramon (95) studied the optimal conditions of fat synthesis in intact tissue by culturing flax embryos in Petri dishes with acetate-1-C^{14}. Optimum conditions for incorporation of acetate by the embryos were 15°C., 200 foot candles of light, pH 6, and 1 to 100 μgm. biotin, depending on the age of the embryo.

Sisakyan & Smirnov in 1956 (96) added acetate-1-C^{14} to chloroplast suspensions which were isolated from green sunflower cotyledons and were incubated for three hours at pH 6.2 with and without added cofactors. Addition of ATP had no measurable effects on incorporation, nor did the addition of Krebs cycle acids. No other cofactors were studied.

A more detailed analysis of fat synthesis in plant material has been described by Stumpf & Barber (97), who prepared particles from avocado fruit mesocarp. These particles readily incorporated acetate into esterified long chain fatty acids. ATP, CoA and Mn^{++} were essential components of the system, and the reaction proceeded anaerobically. Palmitate and oleate

were the principal acids synthesized and short chain fatty acids did not accumulate. No clear-cut source of the reducing capacity for the synthesizing system could be demonstrated. Because of the component requirements for ATP, CoA and Mn^{++}, the isotope distribution data with acetate-1-C^{14} and acetate-2-C^{14}, and the occurrence of the activating enzyme for the reaction: fatty acid + CoA → acyl CoA, it was concluded that the avocado particles possess a system capable of synthesizing fatty acids by multiple β-condensation of C_2 units.

More information became available when water-clear extracts of acetone powders from avocado particles were studied by Squires & Stumpf (98). This system differed from the intact particles in that (*a*) a specific reducing requirement, TPNH (DPNH was ineffective), was now necessary for optimum synthesis, (*b*) the products of synthesis were free fatty acids (oleic together with small amounts of palmitic) rather than esterified acids, (*c*) maximum synthesis occurred only in the presence of a suitable concentration of bicarbonate ($C^{14}O_2$ was not fixed, however), (*d*) citrate did not stimulate synthesis, and (*e*) although acetyl-CoA-C^{14} could be used as a substrate, ATP was still required. Similar observations have been made by Wakil *et al.* with chicken liver enzyme fractions (99).

The acetone powders were stable for a relatively long period at 0°C. Although water extracts lost the capacity to synthesize the free fatty acids, they retained the enzymes which formed the early intermediates.

The possible role of bicarbonate in fat synthesis is of considerable interest, since a regulation of its concentration could be an important factor as a controlling mechanism of fat synthesis. Recently, Squires, Stumpf & Schmid (100) have shown that intact particles of avocado mesocarp are also markedly stimulated by the addition of bicarbonate. Tissue slice experiments, while somewhat variable, also showed an increased rate of biosynthesis of fatty acids in the presence of bicarbonate.

THE CONVERSION OF FAT INTO CARBOHYDRATE AND RELATED TOPICS

The conversion of storage fat into carbohydrate during the germination of a wide variety of seedlings has interested plant physiologists since the middle of the last century. The basic observations of a decrease in the fat content accompanied by a carbohydrate increase and by low RQ values have been described many times. The more physiological aspects of this problem have been adequately reviewed by Zeller (101) quite recently and have not been included in this present discussion.

Only in recent years, however, have we come close to understanding the mechanism of this transformation of fat into carbohydrate. The glyoxylate cycle, a modified tricarboxylic acid cycle, which was described by Kornberg & Krebs (102) in 1957, has provided a pathway by means of which many cell constituents may be synthesized from C_2 units. The reactions of this glyoxylate cycle may be summarized as follows:

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$$\text{Acetyl CoA} + \text{oxalacetate} \rightarrow \text{citrate} + \text{CoA} \quad 1.$$

$$\text{citrate} \rightarrow \text{isocitrate} \quad 2.$$

$$\text{isocitrate} \rightarrow \text{glyoxylate} + \text{succinate} \quad 3.$$

$$\text{succinate} \rightarrow \text{oxalacetate} \quad 4.$$

$$\text{acetyl CoA} + \text{glyoxylate} \rightarrow \text{malate} + 2\text{CoA} \quad 5.$$

$$\text{Sum:} \qquad 2 \text{ acetyl CoA} \rightarrow \text{malate} + \text{CoA}$$

The net effect of one turn of the cycle is the formation of one molecule of a C_4 dicarboxylic acid from two molecules of acetyl CoA. This C_4 acid may then participate in sugar synthesis via phosphoenol pyruvate and the reactions of the glycolytic sequence, or may be used to supply amino acid carbon skeletons via the reactions of the tricarboxylic acid cycle. By such means a wide variety of cell constituents may be formed from C_2 units. This glyoxylate cycle was first demonstrated in *Pseudomonas* KB1 (102) which is capable of utilizing the C_2 compound acetate as its major source of carbon.

Evidence has accumulated in recent years (see p. 200) that the main pathway of fatty acid degradation in higher plants is by means of successive β-oxidations to yield acetyl CoA. Thus, during the early stages of growth, germinating seedlings, in which fat is the main storage reserve being used, represent another class of organisms which are largely dependent upon the utilization of C_2 units for the synthesis of new cell constituents. Kornberg & Beevers (103) have obtained evidence that the glyoxylate cycle is operative in providing a link between fatty acid degradation and sugar synthesis during the conversion of fat into carbohydrate in castor bean endosperms. The pathway of such a conversion may be represented by the following stages: fatty acid+CoA→acetyl CoA→(glyoxylate cycle)→malate→phosphenol pyruvate→triose-phosphate→hexose. Beevers (104) has shown that the distribution of C^{14} within the glucose moiety of sucrose, from experiments in which acetate-1 and -2-C^{14} were fed to slices of castor bean endosperms, was consistent with the operation of this pathway. In addition, Kornberg & Beevers (103) demonstrated that the characteristic enzymes of the glyoxylate cycle, isocitritase and malic synthetase, which catalyze reactions 3 and 5 above, respectively, were present in cell-free extracts from the same castor bean tissue. Aconitase, condensing enzyme and malic dehydrogenase which participate in both tricarboxylic acid cycle variants were also present in these enzyme preparations.

Similar studies have been carried out by Bradbeer & Stumpf (105), who used excised cotyledons from etiolated peanut and sunflower seedlings at the physiological stage in which fat was being rapidly converted into carbohydrate. Acetate-1- and -2-C^{14} were fed to the intact cotyledons and the distributions of C^{14} which appeared in malate and the glucose moiety of sucrose were consistent with the pathway proposed above for the conversion of fat into carbohydrate. From 3-hr. feeding experiments, carboxyl-labeled acetate gave rise to malate which was labeled virtually exclusively in the

carboxyl groups and to hexose which had 75 to 80 per cent of its label in carbon atoms 3 and 4. In contrast, malate with approximately 80 per cent of its label in the two middle carbon atoms and hexose labeled predominantly (over 90 per cent) in carbon atoms 1, 2, 5 and 6 were obtained from methyl-labeled acetate. Isocitritase and malic synthetase, together with fumarase and condensing enzyme, were demonstrated in cell-free extracts from the peanut and sunflower cotyledons (105). In addition, the degradation of the radioactive malate, formed from carbonyl-labeled acetyl CoA and either glyoxylate or isocitrate by these enzyme preparations, provided further confirmation that the incorporation of acetyl CoA into malate was via the malic synthetase reaction.

Carpenter & Beevers (106) have studied the distribution of isocitritase in a wide variety of plant tissues including leaves, roots, tubers, seedlings and fruits. They found that the only tissues which possessed appreciable isocitritase activity were those in which active fat catabolism was taking place. Thus, this enzyme was demonstrated in the seedlings of castor bean, pumpkin, cottonseed, peanut, flax, sesame, soybean, watermelon and sunflower. Isocitritase has also been found in germinating marrow seedlings (107).

Further experiments on the fat into carbohydrate conversions in etiolated sunflower seedlings have been described by Bradbeer (108), who fed $C^{14}O_2$ to the excised cotyledons in the dark. The seedling stage selected was that, determined experimentally, in which fat was being converted into carbohydrate most rapidly. After periods of dark $C^{14}O_2$ incorporation of five minutes and longer the bulk of the radioactivity had accumulated in sucrose. However, when the period of exposure to $C^{14}O_2$ was reduced to five seconds, approximately 80 per cent of the incorporated label was found in malate and most of the remainder was in aspartate and fumarate. Degradation results from a 15 minute experiment showed that the malate was labeled essentially only in the carboxyl groups and the glucose moiety of sucrose was labeled predominantly in carbon atoms 3 and 4. These results have been interpreted in terms of (*a*) β-carboxylation of pyruvate or phosphoenol pyruvate to give β-carboxyl-labeled malate either directly or indirectly via oxalacetate, followed by (*b*) randomization of the C^{14} between the two carboxyls of malate by means of either fumarase or the glyoxylate cycle, and (*c*) the subsequent incorporation of the C^{14} in the α-carboxyl of malate into carbon atoms 3 and 4 of hexose, most probably via phosphoenol pyruvate and a reversal of the glycolytic sequence. This evidence for the incorporation of malate into sucrose in the intact sunflower cotyledons is consistent with the pathway involving the glyoxylate cycle which has been proposed for the conversion of fat into carbohydrate. The significance of the $C^{14}O_2$ incorporation itself is unknown. Clearly it may have resulted either from a net CO_2 uptake by the tissue or simply from such incorporation as presumably occurs when $C^{14}O_2$ is introduced into any system which is normally in equilibrium with an atmosphere containing CO_2. Similar dark $C^{14}O_2$ incorporation into malate and sucrose has also been demonstrated in the cotyledons from

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etiolated peanut seedlings [Bradbeer & Stumpf (109)] and in endosperm tissue from castor beans [Stiller *et. al.* (110)]. It is interesting to note that an active glyoxylate cycle has also been demonstrated in each of these tissues (103, 105).

The metabolism of acetate by higher plants has been discussed by Tokarskaya & Kuzin (111), who observed that acetate-1-C^{14} was absorbed by the roots of two-week-old plants of *Phaseolus vulgaris* and was incorporated into intermediates of the tricarboxylic acid cycle and into amino acids in both the light and the dark, although this incorporation was much greater in the light. Free sugars became radioactive only in the light experiments. The incorporation of labeled acetate into non-volatile organic acids has also been observed in tobacco leaves (112, 113) and tomato fruits (114). In addition, the production of acetate, as determined by the isotope dilution technique, has been demonstrated in tomato stem tissue (50), castor bean, corn, flax, kidney bean, tobacco, geranium, *Bryophyllum*, sheep's sorrel and wood sorrel (115).

It may be relevant to the demonstration of the glyoxylate cycle in germinating seedlings of castor bean and sunflower to note that accumulation of malic, citric, succinic and fumaric acids has been shown to accompany fat breakdown in these tissues (39, 116). On the other hand, it is possible that this accumulation may be the result of the carboxylation of a C_3 acid to give a C_4 dicarboxylic acid by a mechanism similar to that which has been shown to be responsible for the accumulation of di- and tri-carboxylic acids in some succulent plants.

PHOSPHOLIPIDES

A vast literature covers the isolation and identification of phospholipides from plant materials (117, 118). In this section we shall limit ourselves only to investigations pertaining to the metabolism of these compounds in plants.

Hydrolysis

There are several specific enzymes responsible for the hydrolytic cleavage of phospholipides (119). The specific removal of the fatty acid linked to the primary hydroxyl group of glycerol is catalyzed by phosphatidase-A to yield lysolecithin. Phosphatidase-B in turn will now remove the remaining fatty acid residue of lysolecithin (β-acyl-L-α-glycerylphosphoryl choline) to give a glycerylphosphoryl base. Phosphatidase-C will attack lecithin (phosphatidyl choline) to yield phosphatidic acid and choline. This type of enzyme has been found in plants and converts phosphatidyl choline, phosphatidyl ethanolamine and phosphatidyl serine to phosphatidic acid and free base. A fourth enzyme effects the hydrolytic rupture of phosphoryl choline from lecithin with the formation of an α,β-diglyceride. The presence of this enzyme in spinach chloroplasts has been reported by Kates (120).

Of these hydrolytic enzymes only phosphatidase-C has been found extensively in plants. Phospholipides of seeds disappear during germination and reappear as water-soluble choline and other compounds, suggesting that

the role of the enzyme is concerned with the utilization of reserve phospholipides.

Tookey & Balls (121) have found phosphatidase-C in cabbage and cottonseed. From cotton seed it is present in a soluble form and, surprisingly, is not activated by ethyl ether. This may be due to the particulate nature of the cabbage preparation of Kates and the soluble nature of the cotton seed enzyme.

In 1947, Hanahan & Chaikoff (122) observed that the phospholipide isolated from raw carrot had a low nitrogen content and an absence of choline, while that from the steam-heated carrot had a much higher choline content. They postulated that a lecithinase was present in the raw carrot but was absent in the steam-heated carrot. In 1948, Hanahan & Chaikoff (123, 124) isolated a phospholipide-splitting enzyme specific for the ester linkage between the base and phosphoric acid grouping. These observations can probably explain many of the earlier reports of the presence of phosphatidic acids in plant tissues.

Kates (125) has shown that in sugar beet, carrot root, spinach and cabbage leaves the enzyme is associated with the chloroplast fraction. The rate of enzymatic liberation of choline from lecithin is greatly accelerated by saturation with diethyl ether. In the absence of substrate a simultaneous inactivation occurs. Kates studied the kinetics of hydrolysis of glycerolphosphatides by plastid phosphatidase-C (120). He concluded that the primary reaction was the liberation of choline. No activity was observed with phosphorylcholine, lysolecithin, or glycerol-phosphorylcholine as substrates.

A more detailed study concerning the solvent stimulation of plastidine phosphatidase-C was made by Kates & Gorham (126). They suggested that the stimulating solvents such as diethyl ether achieve their stimulatory effects on plastid phosphatidase-C activity by causing substrate and plastid phases to coalesce and that the enzymic reactions actually proceed in the coalesced phase. No enzyme activity appeared in the clear aqueous or ether phases. In addition certain linear aliphatic solvents were readily adsorbed on the surfaces of the lecithin and plastid particles, making the surfaces highly lipophilic and mutually attracting (127).

Biosynthesis

Kennedy has reviewed the studies leading to the current concepts in this field in animals and yeast (128). These ideas are summarized in Figure 3.

Very little work on these lines has been carried out with plant materials. In 1955 Mazelis & Stumpf (129) examined some of the conditions necessary for incorporation of P^{32} into peanut mitochondrial phospholipides. The first step was the esterification of P^{32} into ATP^{32} under aerobic conditions, in the presence of a Krebs cycle acid and Mg^{++}. Only freshly prepared mitochondria incorporated P^{32} into phospholipides and DNP (dinitrophenol) strongly inhibited the reaction. In contrast, when ATP^{32} replaced P^{32} as the phosphate donor, no Krebs cycle acid was required and DNP had no effect. Mg^{++} was still required. The labeled phospholipide has not been identified.

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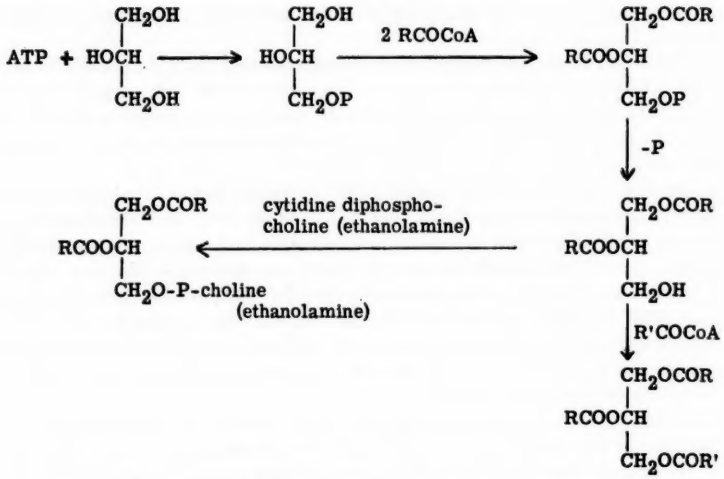

FIG. 3. Current ideas on the biosynthesis of phospholipides and neutral fats in animal tissues.

Since nonradioactive glycerolphosphate had no diluting effects on the incorporation of P^{32} into phospholipides, glycerolphosphate may not participate in the construction of the unidentified phospholipide. The ATP-phosphorylating system was surprisingly stable, since fresh, frozen or lyophilized mitochondria could utilize ATP^{32} in this sequence. This evidence suggested the following:

$$P^{32} \xrightarrow{A} ATP^{32} \xrightarrow{B} \text{Phospholipides}$$

A. TCA cycle acid; Mg^{++}; fresh mitochondria; aerobic; DNP-sensitive.

B. Mg^{++}; lyophilized or frozen mitochondria; anaerobic; DNP-insensitive.

Further work in the biosynthesis of phospholipides in plants by cell-free systems has not been carried out.

Leaf phospholipides.—Kates & Eberhardt (130), in evaluating methods for the isolation of phospholipides from leaves, minimized the action of phosphatidase-C by extraction with hot isopropanol and subsequent chloroform partition, before chromatography on acid-celite columns. Four main fractions were obtained from the extracts of primary leaves of scarlet runner bean plants. Fraction I consisted of inositol-carbohydrate phosphatide, Fraction II was mostly phosphatidyl ethanolamine with some phosphatidyl serine, Fraction III was an unknown phosphatide, probably a peptidyl phospholipide, and Fraction IV was phosphatidyl choline. Because of the inactivity of phosphatidase-C, the presence of phosphatidic acid, which until recently has always been a major component in plant phospholipide frac-

tions, was reduced to a low value. The occurrence of free phosphatidic acid in plant phospholipides is therefore probably an artifact. Eberhardt & Kates (131) next studied the incorporation of possible radioactive phosphatide precursors into these four fractions in leaf tissue for four hours in diffuse light. Their observations are of some interest. The distribution of isotope varied greatly in each fraction depending upon the precursor supplied. On a basis of specific activity (c.p.m./μatom P) the degree of incorporation of $C^{14}O_2$ was in the order: Fr. I and III > IV > II, of pyruvate-2-C^{14} IV > I > II > III, of acetate-1-C^{14} IV > III > II > I, and of $P^{32}O_4$ III > II > IV > I. The variability of incorporation into each fraction by the possible precursors suggested the existence of different routes of synthesis for these phospholipides in leaf tissue. The high incorporation of P^{32} into Fraction III and the small amount of glycerol phosphate in its acid hydrolysis products suggest some close relationship between this fraction and the unknown phospholipide in peanut mitochondria which was studied by Mazelis & Stumpf (129). Of further interest are the recent observations of Westley, Wren & Mitchell (132) of a widely distributed group of phospholipides containing amino acids other than serine.

In the first of a series of publications on plant phospholipides Benson & Maruo (133) chromatographed alcohol extracts of plant tissues in two dimensions and demonstrated the presence of lecithin, phosphatidyl glycerol, phosphatidyl inositol, phosphatidyl ethanolamine, and phosphatidyl serine. Benson postulated that phosphatidyl glycerol may be formed by the interaction of cytidine diphosphoglycerol and 2,3-digylceride. No experimental support was presented.

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THE PINE TREE[1]

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Pines probably appeared on earth at the beginning of the Mesozoic era. The earliest pine fossils date back to the Jurassic period, some 160 to 190 million years ago. Even then pines grew in many places of the Northern Hemisphere. By the lower Cretaceous period, 150 million years ago, pines had already differentiated into two groups: Haploxylon, or soft pines, and Diploxylon, or hard pines. At present, the genus *Pinus* consists of 25 to 30 species of soft pines and 70 to 80 species of hard pines, the exact number depending on how each botanist defines the term "species." All pines so far investigated possess the same number of chromosomes, n = 12.

Pines occur all the way from the long-day cool northern regions, where the growing season is short and the range of air temperature is very great, to the tropics, where days are short, growing season either very long or continuous, and temperatures are relatively high and uniform throughout the year. Altitudinally, pines are distributed from sea level up to 13,000 feet.

Many articles have been written on physiology of pines. We shall endeavor merely to give some examples of work on the physiology of the pine tree. In considering this work, it is important to keep in mind that experimental material from the wild forest is always genetically heterogeneous; the biochemical and physiological performance of pines varies from one individual to another. Also, their slow growth and large size complicate experimental work.

Chemical composition.—Some ten years ago, Erdtmann (25) and his coworkers (70, 71) started investigation of the distribution of chemical compounds, chiefly phenols, in the heartwood of pines. More than half of all the pine species have been investigated.

These studies have shown that the Haploxylon pines are characterized by the presence of: pinetol (a monomethyl ester of inositol); stilbenes and dibenzyls; flavones and flavanones derived from phloroglucinol; and, in the group Strobi, flavones and flavanones derived from methylphloroglucinol. Diploxylon pines contain stilbenes, but not the corresponding dibenzyls; flavanones but not flavones. Pinetol is absent.

Analysis of several hundred individual trees of *P. sylvestris* from different parts of Sweden showed that the content of pinosylvin phenols in the heartwood varies between 0.4 per cent and 1.3 per cent (70). Erdtmann suggested that phenolic compounds possess antioxidizing properties and may possibly

[1] The survey of literature pertaining to this review was concluded September, 1958.

[2] Maintained at Berkeley, California, by the Forest Service, U. S. Department of Agriculture, in cooperation with the University of California.

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play an important part in prevention of autoxidation of terpenes and resin acids.

Traces of the most common heartwood phenols also have been found in the sapwood. Bark of pines also contains phenolic compounds, but these are not the same as the wood phenols; for instance, a flavanol derivative of unknown structure isolated from the bark of *P. ponderosa*, and which is responsible for its yellow color, is not identical with any of the heartwood phenols [Kurth *et al.* (65)].

In 1958 Mirov completed an inventory study of the composition of turpentines of 94 species of pines and summarized the findings in the last of a series of papers (88). The chemical composition of turpentines was found to be specific; in some pines it was rather complicated, and the percentage of each component in a population often varied to a considerable extent. Not all turpentines are composed of terpenes; some contain paraffin hydrocarbons (C_7H_{16} and $C_{11}H_{24}$).

Daugherty in 1954 (19) reviewed the pine oleoresin constituents other than turpentines.

Tallent, Stromberg & Horning wrote a paper dealing with *Pinus* alkaloids (118). From the leaves of *P. sabiniana* were isolated two alkaloids: (+)-α-pipecoline and a new organic base, $C_9H_{17}N$, which was named pinidine. Previously it had been assumed that the genus *Pinus* is alkaloid-free.

Krugman (63) studied anthocyanin and leucoanthocyanins in *P. lambertiana* embryos and seedlings. The anthocyanin was found to be malvin; leucoanthocyanins yielded cyanidin and delphinidin. Anthocyanin was first detected in the hypocotyl of seedlings and later in cotyledons. Within one or two months after germination, the pigment disappeared from the seedling.

Nielson *et al.*, (92) assayed *P. montana* pollen for carbohydrates, fats, mineral elements, and vitamins. Pine pollen was found to contain only half as much protein as angiosperm pollen. Hatano (42) compared the amino acids of pine pollen to those of the megagametophyte. Thirteen amino acids were detected in the protein hydrolysate of the pollen; in addition, proline and histidine were found in the hydrolysate of the female flower, but only these two amino acids occurred free in the pollen.

Mineral nutrition.—The values of nutrients to pines are dependent upon many factors: the nature of the soil [Zinke (133)], climate, the species, and stand conditions. Recent reviews by Leyton (68) and Reuther *et al.*, (97) have summarized some of the problems, results, and implications of studies concerned with nutrient requirements for trees.

Many workers believe that field fertilizer trials are the most expedient way of determining deficiencies [Leyton (67)]. This is not an entirely accurate concept: the site tested is frequently the only place the results apply; or, the results may be misleading for physical-chemical reasons, i.e., the deficiency may be caused by the capacity of the soil to absorb a particular ion, not by a lack of the element in the soil. Therefore, tree physiologists must seek to establish the way the deficiency of a given element is expressed

in a particular species before determining how to manage the forest soil to correct the deficiency.

In pine we now know that elemental deficiencies may result not only in retarded growth and delays in reaching maturity, or in decreasing seed yield [Stoate (112)], but may also increase susceptibility to insects or pathogens. A deficiency of an essential element produces, in pine, characteristic symptoms, in many ways similar to those observed in other plants [Mitchell (90), Pessin (93), Smith (107)].

In treating these symptoms it must be recognized that there is a deficiency range in which tree growth and nutrient concentration are related [Mitchell (90)]; also, additional problems may be involved in evaluating the capacity of added nutrients to correct deficiencies in the field, such as consideration of soil properties and the interaction of ions [Reuther *et al.* (97)]. The exchangeable cations, K^+, Na^+, Ca^{++} and Mg^{++} were found by Zinke (133) to provide a reliable index of soil properties found under a particular species.

The most reliable and accessible index of the nutrient status of a pine appears to be tissue analyses of the needles. In pine, mineral content varies in different parts of the tree crown [Leyton & Armson (69), Guttschick (37)], at different times of the year [Ando (1); Chandler (17)], and in different ages of needles [Leyton (67)]. If the assay is obtained at the end of the growing season, however, a reasonably valid index of the nutrient status and needs of a tree may be obtained (67).

Mycorrhiza.—In a forest, nonpathogenic fungi are commonly found associated with pine roots. This complex, consisting of a plant root and fungus mycelium, is termed mycorrhiza [Boyce (12)]. The roots of pine may be invaded by either ectotrophic or endotrophic mycorrhiza, or both. Mycorrhiza is usually observed in the upper layer of the soil on the short pine roots as swollen, dichotomous branchings of the rootlets. Several recent reviews and discussions have appeared concerning the physiology of mycorrhizal relations in pine [Garrett (33); Hacskaylo (38); Melin (78); Slankis (105)].

The lack of success in establishing exotic conifers has sometimes been traced to the absence of typical mycorrhiza formation by the tree. Thus, by inoculation of nursery beds with soils taken from a vigorous stand of the same species, establishment of the tree has frequently been facilitated [Garrett (32)]. The addition of compost to pot cultures of pine seedlings resulted in a marked increase in mycorrhiza, and increased growth followed [Melin (77)]. The question was raised whether the increased growth was due directly to the addition of the compost with its mineral and organic components or indirectly to the increase of the mycorrhizal associations of the tree [Rayner & Neilson-Jones (96)]. Trees in poor soil seldom grow satisfactorily without mycorrhiza, whereas, in good soil they do grow satisfactorily without it [Björkman (7), McComb (72)].

Experimental evidence in support of different hypotheses of how mycorrhiza benefits pine has been accumulated in recent years, particularly since

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the advent of sterile culture procedures and the use of radioisotopes.

Hatch (45) demonstrated that pine seedlings with mycorrhiza accumulated greater amounts of minerals than those without. He therefore proposed that mycorrhiza was effective by increasing the absorbing surface of the plant roots. Routien & Dawson (99) studied the exchange of ions on fixed particles surrounding the root. They concluded that carbon dioxide released from mycorrhizal roots probably provides exchangeable hydrogen ions, releasing fixed soil cations and permitting an increased absorption by the plant root. McComb & Griffith (73) concluded that the fungus stimulates the root metabolic processes and thereby increases the absorption of nutrients. Kramer & Wilbur (62) verified the theory of McComb & Griffith by studying P^{32} absorption by roots of *P. taeda* and *P. resinosa*. They showed that the mycorrhizal roots accumulated larger quantities of phosphorus than the nonmycorrhizal roots. Nitrate from ammonium salts and amino acids were shown to be absorbed and readily translocated (77, 78).

Slankis (102) reported that the branching of the short roots in pine was caused by some substances produced by the fungus—apparently auxin or its precursor translocated from the fungus hyphae (105). The various degrees of branching may in part be due to the various amounts of the auxins produced by different mycorrhizal mycelia. Besides synthesizing and supplying auxins to the pine roots, and effecting absorption of nutrients, the fungi may also supply vitamins or other metabolites to the host-plant root cells [Hacskaylo (38)]. Additional benefits to pine which have been attributed to mycorrhizal roots are protection from pathogenic fungi [Melin (77)] and drought resistance [Cromer (18)]. Both of these benefits may be indirect, i.e., the result of increased assimilation yielding more vigorous and healthy trees.

It is probable that the fungus also derives additional benefit from the pine. Pine roots supply carbohydrates and additional substances which facilitate the growth of the fungi [Björkman (6, 7); Slankis (105)]. This would also explain the observations that increased light stimulates mycorrhizal development on pine seedlings [Wenger (128)]. Melin & Nilsson (79, 80) demonstrated that the carbon products of photosynthates can be translocated to the fungal associate of the pine mycorrhiza. It is now apparent that many mycorrhizal associations of pine are true symbiotic relationships, of mutual benefit both to the pine host and to the fungal root associate. The growth of a pine tree involves not only climatic and soil environmental factors, but it is also the product of biotic conditions [Hacskaylo (38)].

Photoperiod and thermoperiod.—In 1928 Bogdanov (8) experimented with the effect of photoperiod on pine seedlings of southern origin, grown under natural long days (18.5 hr. in June; 14.5 hr. at the end of August) at 60° of latitude. The seedlings completed their growth in July; then the terminal buds opened again, so that two flushes of growth resulted during one season. In winter the seedlings suffered from low temperatures.

Seedlings from the same localities, grown under 9-hr. photoperiods, i.e., under their natural short-day conditions, developed normally and were not

damaged by cold. This experiment was apparently the first attempt to apply the concept of photoperiodicity to forest trees, including pines.

In 1936 Kramer (57) began to experiment with photoperiodic reactions of *P. taeda* seedlings. He found that under days shortened to 8.5 hr. the seedlings made slightly less growth than under normal days (of 14.5 hr. in June and 9.5 hr. in December). Under 14.5-hr. photoperiod or in continuous daylight the seedlings grew well throughout the year.

In 1941 Phillips (94) reported that loblolly pine seedlings grew more rapidly under long days produced by the addition of red irradiation (680 to 1,400 mμ) than under other experimental conditions (i.e., blue light or darkness). Under supplementary blue light a stunting effect resulted.

In his researches Wareing expanded knowledge of the influence of photoperiod on pine seedlings a great deal (125, 126). His experimental pine was (and still is) *P. sylvestris*.

Under a short photoperiod of 10 hr. as opposed to a long photoperiod of 15 hr., duration of growth of the first-year seedlings was reduced, fewer needles were formed, and the length of intervals and needles was reduced. Root/shoot ratio and appearance of roots were not affected.

Maximum growth of the seedlings was attained at the 20-hr. photoperiod. The growth was considerably less under continuous light. The rates of leaf production and stem elongation were greatly accelerated by the introduction of one 4-hr. dark period, as compared with continuous illumination; when two 4-hr. dark periods were introduced, there was a further increase in leaf number and in stem elongation.

Cambial activity of one-year-old seedlings was maintained longer under a 15-hr. photoperiod than under a 10-hr. photoperiod. It was concluded that in the fall, when daylight is reduced to 10 hr., cambial activity ceases. Nutrition was also apparently involved in the cessation of growth. In the spring, cambial activity begins when days are as short as in the autumn.

It is perhaps pertinent to mention here Chailakhian's suggestion (16) that photoperiodic response is a complicated phenomenon and that it involves many other factors besides the length of light hours.

Wareing suggested (126) that two systems seem to operate in pine seedlings during the dark period: (*a*) a growth hormone, formed during the preceding light period, is active during the first hours of darkness; and (*b*) a growth inhibitor becomes effective after four hours of darkness.

Zahner (132) experimented with the effect of interrupted photoperiod on pines. Control seedlings of *P. taeda* were grown under 9.5 hr. of daylight followed by 14.5 hr. of darkness. These were compared with the seedlings whose dark period was interrupted in the middle by 30 min. of 200-watt light. The seedlings with the interrupted night made more than twice the height growth, began growing two to four weeks earlier, and continued growth to September, while control seedlings "almost ceased to grow early in June." Apparently, in Zahner's experiments, as Wareing had suggested, a growth inhibiting substance was formed during the dark period. The inhibitor was

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unstable at the beginning of the dark period and was destroyed by light.

Some of the early studies, including many not discussed here, had definite shortcomings. Equipment was often poor, the effect of temperature was not isolated from that of light, and quality of the light was rarely considered. When seedlings were subjected to long photoperiods, the effects of photoperiod were probably confounded with those of photosynthesis. The number of experimental plants was often very small and variation in individual response was often great. [Vaartaja (123) was one of very few investigators who mentioned the individual variation of pine seedlings in their reaction to light.] Most experiments were not statistically designed.

Downs & Borthwick (24) fully realized the shortcomings of previous (though very valuable) work, when they started to experiment with seedlings of (chiefly) *P. sylvestris*, and also *P. virginiana* and *P. taeda*, using much better light and temperature controls. They demonstrated that normal seasonal height growth in the seedlings consists of elongation of elements initiated in the bud the year before. Under long photoperiods, however, the new structures elongate as fast as they are formed, so at a 14-hr. photoperiod the growth "is almost continuous." At a 16-hr. photoperiod growth was somewhat erratic; the rate of growth was perhaps even faster than at 14-hr. daylight, but they noticed "several periods of growth stoppage," possibly caused by disturbing the balance between initiation of structures and their elongation.

In nature, terminal buds were formed in the fall when days become short; but in the spring the buds became active "even though the day length may still be shorter than that which induced their formation;" the low winter temperature caused the changes necessary for resuming growth, i.e., here, thermoperiod was involved—a need for low temperature previously reported by Gustafson in 1938 for seedlings of *P. resinosa* (36).

All these photoperiod studies with pines dealt with vegetative growth of very young seedlings. How photoperiod affects older pines we do not know. Nor do we know how different photoperiods or combinations of photoperiods affect the flowering of pines. Righter (98) reported that some pines produce pollen catkins and female conelets very early, sometimes at the age of two or three years. Mergen & Cutting (83) reported development of pollen catkins on one-year-old seedlings of *P. montana*. Generally, however, pines reach the reproductive stage much later in their life.

So far, however, no initiation of female "flowers" has been induced by any artificial methods on pines which have not already reached the flowering stage. After this stage, however, seed production has been induced by such practical measures as girdling [Hitt (48)] or fertilizing [Schubert (101)].

In 1956 Mirov (87) surveyed many northern and southern pines growing in California at a latitude of 38° and found that, when northern (long-day) pines were moved to the southern (shortened-day) locality, their flowering habits were not changed. Similarly, when short-day, tropical pines were moved north, where summer days are long, their flowering habits were not

changed. Wareing (127) also could not find any evidence that photoperiod controls reproduction in pines.

It appears, then, that as far as flowering under natural conditions is concerned, pines are neither short-day plants like poinsettia nor long-day plants like spinach, but are neutral.

Karschon (52), we believe, was the first to apply Went's term "thermoperiodicity" (129) to pine seedlings.

Precise experiments, in which thermoperiod is divorced from photoperiod and in which thermoperiod itself is accurately controlled, have been possible only recently since modern laboratories became available. Kramer (58, 59) studied the effect of day and night temperatures on the height growth of *P. taeda* seedlings in the air-conditioned greenhouses of the Earhart Plant Research Laboratory (130). Best growth was made with the widest spread between day and night temperatures, and poorest growth when nights were as warm as days.

During the second year of experimentation, Kramer (59) found that an increase of day temperature was accompanied by increased shoot growth, but an increase in night temperature was accompanied by decreased growth; warm nights which tended to reduce the length of the growing season were unfavorable to seedling growth. Height growth was the same at 30°–17°C. and 23°–11°C. day-night temperatures; Kramer therefore demonstrated that the difference between day and night temperatures was more important than the actual temperature, and he suggested that this relation between day and night temperatures may explain the southern distribution of some pines. For instance, *P. strobus* occurs in the mountains of Georgia where days may be hot but the nights are cool; it does not grow in the Piedmont at the same latitude where nights are warm. The same phenomenon was observed by Kramer in *P. taeda* (58), so it appeared that this rule held for all pines. But Denevan's studies (23) in Nicaragua, where pines reach their southern limit, have shown that *P. oocarpa*, a mountain species (growing under warm day-cool night conditions), and *P. caribaea*, a coastal pine (growing in warm-day-warm night environment), reach about the same latitude, the latter species even extending slightly farther south. Apparently the southern limit of these two pines is determined by conditions other than the relation between day and night temperatures. Of course these might have been specific differences.

Sarvas (100) reported the effect of light on germination of *P. sylvestris*. Germination started two days earlier, and was far better in light than in darkness.

According to Borthwick (10), Downs & Toole reported that "in certain species of pines a long photoperiod induced no higher percentage germination than did a few minutes of light given at both ends of an equally long period of darkness."

Toole *et al.* (121) reported that only 10 per cent of *P. taeda* seed germinated in darkness, while 90 per cent germination was obtained when the

combination of light and imbibition was favorable. Red light was found to induce germination while far-red radiation inhibited it; the inducement and the inhibition can be done repeatedly. In their tests, about 50 per cent of the seed germinated after a single 16-min. exposure to red light following one to four days of imbibition. As the period of imbibition increased to 64 days, progessively greater percentages of seeds germinated in response to the same 16-min. red light treatment.

Photosynthesis.—Many notable contributions to the study of the relation between light intensity, rate of photosynthesis, and respiration in pine have been made by Kramer and his associates (60, 61, 76). The rate of photosynthesis in pine trees was found to increase up to the maximum intensity of applied light. This is interpreted as indicating that light penetrated into areas of the tree not reached at the lower intensities.

The effect of low light intensity on pine has also been studied. Decker (22) found that the photosynthetic rate in *P. sylvestris* seedlings increased linearly up to 6400 ft. c. but the rate declined more steeply, nonlinearly, below 1800 ft. c. The ability of pine to photosynthesize and accumulate dry matter at low levels of light intensity has been related to the capacity of certain species to establish themselves successfully in the shade of a forest stand [Bormann (9)].

The photosynthetic action spectrum in pine has been observed to have a maximum at 5461 A and 5780 A. This is more in the blue region than the maxima reported for angiosperms [Burns (14)]. Other common factors which have been studied in relation to the photosynthetic rate in pine are temperature [Decker (20); Freeland (28); Tranquillini (122)], carbon dioxide availability [Decker (21)], and age of the needles [Freeland (29)]. Pines have been shown to photosynthesize in winter at temperatures near 0° C. (28, 122). However, the actual net photosynthetic rate will depend on concomitant respiratory and translocation rates and the CO_2 concentration. Recent studies of McGregor *et al.* (76) throughout a growing season indicate that the maximum efficiency of photosynthesis per unit of needle fascicle length occurred in July for *P. strobus* and in May for *P. taeda.* However, in expressing apparent photosynthesis in units of pine needles, it must be recognized that rates of photosynthesis vary in different age needles [Freeland (29)]. A maximum rate is attained after needle expansion in the first year and a steady decline occurs in two- and three-year-old needles.

Bernard-Dagan (5) followed the diurnal and seasonal variations in carbohydrate content of needles of *P. maritima.* He found that the carbohydrate level was higher in June than in November and that the diurnal pattern was related to variations in light intensity.

Growth, auxin, and reproduction.—Different species of pine have different periods of growth, even when growing at the same site [Fowells (27)]. Kienholz (53) found that pine roots were first to begin growing in early spring, followed by the leader, then the cambium and needles. He attributed the

cyclic periodicity of pine growth to a depletion of food reserves in the tree. MacDougal (74), in dendrometric studies of *P. radiata*, also determined the time of growth inception. He concluded that neither temperature nor moisture was a primary cause of the beginning of growth in pine, but that an inherent quality of the tree was the key factor. Attempts have been made to increase the growth of pines through inducing polypoloidy by treatment with colchicine [Mirov & Stockwell (89)]. However, polyploid pines have invariably been dwarfed, malformed, or slow growers [Komissarov (54)].

No relationship was found between the amount of height growth of conifer seedlings and the length of the growing season [Kozlowski & Ward (55)]. The distribution of growth hormone in shoots of two species of pine was described by Mirov (85). A gradient existed in the pine shoots, the maximum concentration occurring near the shoot base. Brown (13) has shown that the auxin physiology of the *P. palustris* shoot apex is probably related to the inhibition of growth at the early seedling stage, resulting in the so-called "grass stage." Fransson (30) has found pine shoot auxin to be chromatographically different from β-indoleacetic acid. However, the recent discovery of many auxin substances or precursors such as indoleacetonitrile and indoleacetaldehyde indicates the need for reevaluating these older studies of growth substances in pine.

The natural level of auxins in pine roots has not been determined, although many workers have applied auxin to pine stem cuttings to facilitate rooting [Snow (108)]. Slankis (103, 104) has had considerable success in stimulating the formation of mycorrhizal-like branchings and growth of excised pine roots in culture by the addition of various synthetic auxin compounds. It is probable that the interaction between auxin and antiauxin-like substances or inhibitors may be more important than food reserves for growth initiation and development [Thimann (119)]. A weak foliar spray of an antiauxin, maleic hydrazide, was shown to inhibit growth of *P. banksiana* and *P. sylvestris* [Rai & Hamner (95)].

The work of Camefort (15), who demonstrated that the concentration of ribonucleic acid in pine shoots is highest in the dividing cells, offers interesting opportunities for further study.

Translocation.—The problems of translocation in trees have been reviewed at the two recent symposia of the physiology of forest trees (117, 120).

The movement of water to the tops of trees is usually explained on the basis of the Dixon-Joly water-cohesion theory. This theory found support in MacDougal's studies of the movement of solutions in *P. radiata* (75). Recent work with radioisotopes [Fraser & Mawson (31)] and dyes [Vité (124)] in pines indicate that the upward movement of mineral elements, presumably in the water stream, is not straight up the tree, but often follows a narrow spiral-like path with very little lateral movement. The nature of the path of upward movement appears to depend upon the species and age

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of the tree. The importance of the water diffusion-pressure-deficit in moving water into and within a pine is indicated by studies of Slatyer (106) and Stone *et al.* (115).

Studies of the movement of mineral solutes in pine have been greatly facilitated through the use of radioisotopes. P^{32} was shown to move more rapidly across the lateral roots than in the stems of *P. taeda* seedlings [Moreland (91)]. The nature of the root cells and their active protoplasmic stream versus that of the stem might help explain such observations. However, other factors are also undoubtedly involved. Ferrell & Hubert (26) compared the rates of movement of Ca^{45} and P^{32} in pole-blighted and healthy *P. monticola* trees. They found that the radioactive elements moved more rapidly in the blighted than in the healthy trees. This fact was attributed to the higher rate of transpiration in the diseased trees.

The paths of translocation and accumulation of elements in pines is an active area of study. Wikberg (131) found that shoots of *P. sylvestris* which absorbed P^{32} through the roots accumulated most of the activity in meristematic regions in phloem and cambium. The concentration was considerably lower in the xylem and cortex. This raises the question of whether or not the xylem is the main stream for translocation of all elements. Since phosphate is often bound to sugars, it is quite possible that movement of this element, at least, is predominantly or partly in the phloem. Melin & Nilsson (81), in studies of mycorrhiza-infected *P. sylvestris* seedlings, demonstrated that carbohydrates moved from the plant to the root fungi. They also showed (82) that transpiration rates of the seedling markedly affected the rates of movement of phosphates (P^{32}) from the fungus to the host tissue.

Seed germination, respiration, and involved enzymes.—Gross respiration, respiratory quotient, and oxygen absorption have often been used as indices of the substrates being utilized by germinating pine seeds [Stone (113); Hatano (43)]. However, the respiratory variations within pine seeds from different geographical sources [Huart (49)], and variations with water content and physiological maturity of the seeds [Hatano (44)], render interpretation of such measurements questionable. A similar difficulty was also faced by Kozlowski & Gentile (56), in attempting to measure the respiration of pine buds. Stone (114), in his researches with germination response of Jeffrey pine seed at different temperatures, suggested that studying the inherent changes in substrates, enzymes, or cofactors occurring at 5°C. and 25°C. would be a more promising approach to seed problems.

The goal of such studies has usually been merely to provide a more rapid method of overcoming the refractory nature of germination and assure rapid initial growth of the pine seedling. At present, chilling (commonly called stratification) of pine seed at 5°C. for 3 months [Mirov (84)] or alternating between 2° and 25°C. [Asakawa (2)] is the recommended practice. Embryo excision is occasionally used [Haddock (39)].

Mirov (86) studied the changes in fatty acids in sugar pine and Jeffrey pine seeds during stratification. The unsaturated fatty acid, linolenic, de-

creased more rapidly in *P. lambertiana* during storage than in *P. jeffreyi* and disappeared during stratification. Kaloyereas (50) recently applied a rancidity index to loss of seed viability in *P. palustris;* he also concluded that fat changes can be correlated with loss of germinability. Studies of Boudon (11) on the site and extent of lipolysis in germinating *P. maritima* seed indicated that most fat oxidation occurred in the embryo rather than in the endosperm, which initially had the higher fat content. However, the specific nature of the hydrolytic or oxidative changes indicated in these studies and the cause of loss in germinability must still be ascertained.

Baron (4) found in studies of the amino acid and sugar pattern of germinating *P. lambertiana* seed that the embryo tissue contained the same amino acids as the endosperm, with arginine and the amide, asparagine, being present in the largest amounts. Initially, monosaccharides were absent in the ungerminated embryo, and sucrose, raffinose, stachyose, and inositol were present. Inositol, glucose, and fructose markedly increased during germination.

In detailed studies of germinating seeds of *P. thunbergii*, Goo & Negisi (34) followed quantitative changes in the fats, total nitrogen, sugars, and starch. They concluded that nearly all nitrogen-containing compounds moved from the endosperm to the embryo during germination. The detailed analysis of the amino acids in germinating *P. thunbergii* seeds by Kano & Hatano (51) was supplemented by Hatano's study of the keto-acid changes (41). Only two amino acids were initially found in whole ungerminated seeds, whereas 16 were recognized in the seedling after germination and absorption of the endosperm. Six amino acids that appeared on the chromatogram could not be identified, indicating fruitful possibilities for further studies.

The many chemical changes observed in stratified and germinating pine seeds indicate that the more readily oxidized, low-molecular weight substances derived from fats, proteins, and carbohydrates are mobilized and accumulated during the pregermination period. These substances provide the necessary substrates and energy essential for the rapid cell division and growth which must occur during the initial stages of germination. Successful pine seed germination probably depends primarily upon the amounts and kinds of constituents available and the capacity of the embryo to utilize them.

The variations in chemical constituents can frequently be correlated with cellular enzyme levels and activities. Hatano (41) interrelated the many observed changes in amino acids by postulating the presence of transaminase and amide-producing enzyme systems in germinating pine seeds. Transaminase was demonstrated in pine tissues in 1947 by Leonard & Burris (66). A very active arginase system [Guitton (35)] and also α- and β-galactosidases [Hasegawa *et al.* (40)] have been demonstrated in germinating pine seeds. In germinating *P. thunbergii* seeds, Hattori & Shiroya (46) found the invertase activity increased markedly while the galactosidase activity diminished. Stanley & Conn (111) working with cell-free particulates from *P. lambertiana*

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seeds and seedlings, observed diminishing levels of activity of Krebs cycle oxidative enzymes associated with the mitochondria during germination, or when the seeds were stratified for 60 and 120 days.

Germinating pine pollen has also been used in many recent pine enzyme studies. Phosphohexokinase was demonstrated [Axelrod *et al.* (3)], and evidence for the glycolytic pathway was found in *P. ponderosa* pollen [Hellmers & Machlis (47)]. This anaerobic pathway has been shown to be supplemented during pollen germination, in part, by the aerobic hexose-monophosphate pathway [Stanley (110)]. Seasonal variations in extracellular enzymes of the phloem and cambium of *P. sylvestris* were reported by Kuprevich (64) as follows: α- and β-amylases are the most prominent; catalase, phenolase, and tyrosinase activity diminished in September; while the amylase, invertase, and urease activities of the extract remained quite high. The capacity of the plant to hydrolyze oligosaccharides and other molecules potentially involved in translocation, may possibly be significant in determining what substances move in and out of the cells and in explaining seasonal fluctuations in their movements.

Enzymatic studies in pine hold particular promise of reward because of the capacity of pines to produce or accumulate certain specific chemical constituents. The terpene constituents of pine are one such group of compounds [Mirov (88)]. Recent studies have indicated that the terpenes are metabolized in the growing pine tissues [Sukhov (116)] and that they may possibly arise through the sterol precursor, mevalonic acid [Stanley (109)]. The search for the enzymes responsible for the production of the many varied chemical compounds characterizing the pines has just begun, and it promises to be a challenging opportunity for future study.

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PHYSIOLOGY OF VIRUS DISEASES[1]

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In volume 3 of the *Annual Review of Plant Physiology*, it was concluded that analogies between virus multiplication and either the growth of bacteria or the conversion of precursors into enzymes were false and, further, that the picture of infecting particles directly replicating to give a uniform end product was also inaccurate. Then current evidence suggested that a more useful concept was that plant virus diseases are primarily disturbances in the host's nucleoprotein metabolism (1). Since then this concept has been supported by much new evidence, of which probably the most significant is the discovery that the characteristic nucleoprotein rods of tobacco mosaic virus (TMV) are not, as they were long thought to be, the minimal infective units.

THE ACTIVITY OF VIRUS FRAGMENTS

The first claims that TMV retained some infectivity after being disrupted by sodium dodecyl sulphate (2) or phenol (3) and that the infectivity is not conferred by residual virus particles are established unequivocally (4, 5, 6). Such preparations are free from the characteristic rods and from detectable protein that is serologically related to the virus. In addition, the instability of the preparations indicates that the infectivity no longer resides in intact virus particles. Such disrupted preparations consist largely of nucleic acid, and their inactivation by ribonucleases shows that nucleic acid is essential for infectivity. Indeed, the simplest explanation of current results is that nucleic acid provides the whole of the infective component, but until the infectivity of the nucleic acid preparations has been considerably increased by methods other than combining them with virus protein, it will be impossible to exclude the possibility that infectivity also demands the presence of some other small constituent. The fact that recombining the nucleic acid with virus protein increases infectivity (7) is not wholly relevant to the point. Although this is most reasonably interpreted as meaning that reconstituting the nucleoprotein rods protects the nucleic acid from hazards encountered during the infection process, it could also mean that the added protein contains more of the small constituent that can be postulated as essential in the nucleic acid preparations.

Whether or not nucleic acid per se is infective, and the evidence suggests that it probably is, it is clear that the production of TMV does not demand the presence in host cells of an initial nucleoprotein particle to act as a "starter," "model," or "template" for a continuing process of replication. The only part of the virus that there is any need to consider to be self-replicating is the nucleic acid, which not only produces more of itself, but

[1] The survey of literature pertaining to this review was concluded July, 1958.

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also sets up conditions that lead to the synthesis of the appropriate protein with which it then combines to give completed virus particles. As with bacteriophages, with which the idea of nucleic acid being the only replicating component and sole carrier of genetic information was first mooted (8), the formation of TMV seems to be a final assembly process, essentially different from multiplication, in which the components that have already either multiplied or been synthesized are brought together in a stable form.

This assembly process can in part be duplicated *in vitro*. Most methods used to disrupt TMV particles denature the protein, but appropriate exposure to alkali produces soluble fragments with a weight equivalent to a molecular weight of about 90,000, which when brought to below pH 6 aggregate to form rods resembling particles of TMV (9, 10, 11). These rods are unstable and disaggregate when the pH is raised to 7, but when the protein is aggregated in the presence of nucleic acid, the nucleic acid is incorporated in the rods, which then remain intact at pH 7 (12). Thus both components of complete virus particles seem to fulfil a structural purpose, the protein to protect the nucleic acid from inactivation, and the nucleic acid to hold its protein covering firmly together. The reconstitution of particles with the form and gross chemical composition of TMV has been stated to confer infectivity on mixtures of the two components neither of which previously possessed it (12, 13, 14), but the evidence for this is equivocal. Most of the results suggesting that infectivity has been conferred by reconstituting particles can be interpreted in other ways, either because the treatments used removed already infective particles from protein that inhibits infection, or because infective fragments, which otherwise would have become inactive before or during testing, were stabilized (6).

If the only purpose served by the virus protein is to protect the nucleic acid from inactivation, the protein from different strains of TMV seems to possess different protective abilities. The rib grass (plantain) strain is much less infective, weight for weight, towards *Nicotiana glutinosa* than is the type strain, but the infectivity of nucleic acid preparations made from the two is similar, and when nucleic acid from the rib grass strain is combined with protein from the type strain the resulting particles are stated to be more infective than the original rib grass strain (15). Clearly then, different proteins aid the establishment of infection to different extents; as they also protect against inactivation *in vitro* by ultraviolet radiation to different extents (5), there is no need to look for any explanation other than that they amount to escorts of different efficiencies. There is also, however, the obvious possibility that they play some more active part in initiating infection. They may, for example, serve to attach particles to infection sites, as bacteriophages are attached to bacteria, and proteins from different strains may do this more efficiently in some host cells than in others. The fact that both intact virus particles inactivated by ultraviolet radiation (16) and the small protein frag-

ments produced by disrupting TMV with alkali (6) inhibit infections by active virus, is plausibly explained by postulating competition for infection sites. Such sites could exist on plastids, microsomes, nuclei, or other cell components, but there is no direct evidence that they do, and if the nucleic acid needs to enter such a cell component as a preliminary to multiplying, the presence of combined protein seems helpful rather than essential.

Protein fragments, which contain little or no nucleic acid and resemble the fragments produced by disrupting TMV with alkali, occur in sap from infected plants (17, 18, 19, 20, 21). These, like the small nucleoprotein fragments described earlier (22) and which for unknown reasons no longer occur in the quantity they did some years ago (21), are antigenically similar to infective virus and aggregate to form the characteristic rods when treated in various ways. When aggregated in the presence of TMV nucleic acid they combine with it (4). These small anomalous proteins do not become detectable until infected plants already contain considerable amounts of virus, and their amount is correlated with the total amount of virus while this is increasing rapidly, but after that it fluctuates erratically. The biological significance of these protein-only particles is uncertain, but experiments with plants supplied with $C^{14}O_2$ when the virus was increasing rapidly suggested that they are short-lived intermediaries in the synthesis of virus particles (23). Similar experiments with plants infected with turnip yellow mosaic virus led to the same conclusion about the protein-only particles that occur in such infections (24). The idea, however, that they are precursors of nucleoprotein has been challenged because the ratio of nucleoprotein to protein particles remains constant at 2:1 throughout prolonged periods of infection and in a wide range of conditions that affect the total quantity of anomalous particles (25).

Even if some of the protein particles are intermediaries in the synthesis of nucleoprotein particles, not all need be; some may be side effects of the aberration in protein metabolism and others may come from disrupted nucleoprotein. In this context it may be significant that the only method whereby the ratio of protein-only to nucleoprotein particles in extracts from plants infected with TMV has been increased is by keeping plants for periods at 37°C., conditions in which the nucleoprotein diminishes in quantity (21). Nucleoprotein metabolism is a dynamic process, with the amount of finished product at any one time representing the balance between anabolism and catabolism; whether the finished products, anabolic intermediaries, or catabolic fragments are detected will depend primarily on their stability in cells and in extracts made from them. Leaf extracts contain ribonuclease and other substances that readily inactivate virus nucleic acid, whereas the leaf proteases do not readily hydrolyse virus proteins. As the proteins are also readily identified serologically, it is not surprising that these rather than free virus nucleic acid have been most often reported; however, by grinding at low

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temperatures (liquid nitrogen) leaves in which TMV was increasing rapidly, extracts have been made that are stated to contain infective nucleic acid (26).

Whatever the origin of the various anomalous particles occurring in extracts from virus-infected plants, it becomes increasingly obvious, as more diseases are studied in detail, that it is usual rather than exceptional for such extracts to contain not one type of specific particle, but a range of types related to one another in different ways. In addition to the variations in particle length that occur with all elongated viruses, and the protein-only particles already mentioned in infections with TMV and turnip yellow mosaic, more than one specific product has been identified in plants infected with tomato bushy stunt virus (27), potato virus X (28), and the Rothamsted tobacco necrosis virus (29). The occurrence of noninfective nucleoprotein particles of the tobacco necrosis virus can be attributed, though not necessarily correctly, to the fact that infected leaves contain systems that inactivate it without disrupting it (29).

THE INFECTION PROCESS

By analogy with the behaviour of bacteriophages, it is reasonable to look for evidence that the nucleic acid separates from the protein of plant viruses as an early step in the infection process, perhaps as a preliminary to the nucleic acid entering some host-cell component in which it is protected from inactivation and is able to exert its influence on the cell's metabolism. There is nothing conclusive to show that this happens, but there is much evidence compatible with the idea, and it can be taken as established that viruses change their state and become unstable soon after being inoculated into leaves. For example, exposing leaves to ultraviolet radiation makes them temporarily resist infection by viruses that normally infect them readily, but even such stable viruses as TMV fail to survive through this period of a few hours and do not proceed to multiply when visible light has restored the initial susceptibility of the leaves (30). Also, experiments on the photoreactivation of potato virus X inactivated by ultraviolet radiation show that these particles change their state at least twice within a few hours; the first change, which at 20°C. a few undergo within 15 min. but most need 30 min. and some more than 1 hr., makes them able to be reactivated by visible light acting through some host system; the second change happens within an hour or two of the first and is to a state in which light no longer makes them infective (31). This period, too, is approximately the same as that during which pancreatic ribonuclease prevents infection when rubbed over the inoculated leaves. This enzyme is a powerful inhibitor of infection by all plant viruses yet tested and there is no better explanation of its action than that it destroys the nucleic acids when they separate from their protein wrappings (32), for it does not damage intact virus particles. The only objections to the explanation are that some other substances that have no ribonuclease ac-

tivity are also powerful inhibitors of infection (33) and leaf ribonuclease does not have the same action.

Other evidence of change in the status of infecting particles comes from exposing leaves to ultraviolet radiation at different intervals after inoculation with viruses. For a time, which varies with different viruses and virus strains (34, 35), the resistance of the virus to inactivation remains unchanged, but it then increases and continues to increase until irradiation no longer prevents the development of a lesion. The final increase to complete resistance to inactivation presumably marks the time at which infection is not only established, but has developed through a full cycle of virus production, and new virus has moved from the epidermis into deeper cells where it is no longer reached by the radiation.

The early changes are the more important for our present discussion. The first increase in resistance is most plausibly explained by postulating that the virus particles have now become partially protected against ultraviolet radiation by becoming associated with cell components that also absorb the radiation. That this association may be accompanied by or require the separation of virus nucleic acid from its protein, is suggested by the results of experiments with different strains of TMV inoculated both as intact virus and as nucleic acid (36). With intact virus as inocula, different strains take different times for their resistance to inactivation to start to increase, but not with inocula of their nucleic acids, which show the increased resistance without the preliminary lag period that occurs with intact virus. It may be significant, or simply coincidental, that a strain with a short lag period *in vivo* is also more rapidly separated *in vitro* into protein and nucleic acid by sodium dodecyl sulphate than one that has a long lag period (36). That nucleic acid, although weight for weight a less effective inoculum than intact virus, gets a head start is further suggested by the claim that multiplication of TMV becomes detectable some hours earlier in tobacco leaves inoculated with nucleic acid than with intact virus (37). The continuing increases in resistance of infecting virus to inactivation by ultraviolet radiation can be interpreted as evidence that the nucleic acid, now established in some cell component, is exerting its influence and stimulating the synthesis of absorbing substances that will go to make more nucleic acid. The nature of the inactivation curve changes shortly before irradiation ceases to prevent infection and this may indicate the first appearance of new infective material (35), but whether in the form of free nucleic acid or as nucleoprotein is unknown.

Neither is the form known in which the new virus spreads from cell to cell within leaves; the first crop of virus, however, passes unnoticed in infectivity assays and it is only at the time when some hundreds of cells at each infection site have become infected that multiplication becomes detectable (38). From then on, except in hosts in which a virus is strictly localized in necrotic lesions, the virus content of successive extracts made from inoculated leaves increases rapidly, partly because for some hours the virus content of individ-

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ual cells continues to increase, but mainly because increasing numbers of cells become invaded. Presumably the same cycle of events is repeated in each newly invaded cell as in cells infected directly at the time of inoculation, but on this there is no information.

Infection of the first lots of cells by TMV in inoculated tobacco leaves does not lead to chemical changes presently detectable in leaves or their extracts. However, some days after inoculation, when the virus content of successive extracts is increasing exponentially, both insoluble protein and nucleic acid are reported first to increase and then to decrease as the amount of virus obtained in extracts increases (39). There are reports that the normal leaf nucleoproteins decrease in amount proportionally to TMV production (17, 40), but these have not been confirmed (28, 41). The concentration of normal leaf proteins varies considerably over short periods, suggesting that they are readily metabolized, but there seems to be no consistent correlation between them and either the amount of virus produced or the severity of symptoms evinced by leaves. Neither potato virus Y, which causes a disease comparable in severity to tobacco mosaic, nor tobacco etch, which causes a much more severe disease, usually affect the concentration of the normal proteins in sap, though they sometimes decrease it slightly; neither virus reaches amounts in sap large enough to be detected electrophoretically. Potato virus X and TMV, both of which reach amounts readily detectable by electrophoresis, sometimes decrease the concentration of normal proteins, usually in young rapidly growing plants which favour a high virus content, but even in these conditions the concentration of normal proteins is sometimes unaffected. Simultaneous infection with these two, each of which then reaches as much as when on its own, has no more effect on the concentration of normal proteins in sap than infection with one or other alone (28).

Viruses will multiply in detached pieces of leaf not supplied with nitrogen. Under these conditions the normal proteins break down and the virus presumably is synthesized from these breakdown products. This, though, seems to be the only sense in which the normal proteins act as a forerunner of viruses. As no plant viruses have yet been reported to contain any unique constituents, their synthesis could well occur from the normal pool of cellular amino acids, purines, and pyrimidines, but it seems that the formation of TMV protein may begin at even lower levels than this, with the incorporation of ammonia into products from which the virus is ultimately assembled (42, 43, 44). That the synthesis of TMV nucleic acid starts from the normal pool of purines and pyrimidines is suggested by the fact that this is depleted when the virus is multiplying rapidly (45), and by the fact that analogues, such as thiouracil (46) and azaguanine (47), are incorporated into virus particles when they are supplied to leaves in which the virus is multiplying.

THE INFLUENCE OF HOST METABOLISM ON VIRUS INFECTIONS

The concept of virus diseases as disturbances of the host's nucleoprotein metabolism almost necessitates the conclusion that the ability of viruses to

infect, multiply, and cause symptoms will vary greatly with changes in the physiological condition of the host. There is abundant experimental evidence of such variations. Almost every change in growing conditions that affects plant growth or cellular metabolism also affects the behaviour of viruses, but although it is easy to establish phenomena, it is more difficult to explain some of them.

The increases in susceptibility to infection when plants are raised under low light intensities (48), or given abundant supplies of water (49) or nitrogen (50), are readily explained by postulating that such treatments increase the succulence of leaves, which therefore are more readily injured at inoculation and so produce more wounds through which infecting virus particles can enter. But whether this is the true or the whole explanation is far from certain, especially as many viruses seem to spread more readily from cell to cell in such succulent leaves than in tougher ones, though this can hardly depend on injury at inoculation or the thickness of the cuticle. Also, a day or so in darkness before inoculation will increase the number of infections produced by a given inoculum, though this treatment does not noticeably affect the susceptibility of leaves to injury during inoculation (51). Similarly, although the increases in the concentration of TMV produced by changes in nutrition that increase plant growth (52, 53, 54) are what might be expected from treatments that encourage protein sysnthesis, something more specific is needed to account for the fact that concentrations are greater in leaves deficient in Mn than in those well supplied (55).

Exposing plants to temperatures around 36°C. also produces effects that seem to demand as explanation some specific interactions between host components and viruses. Effects on susceptibility to infection are strikingly different when plants are exposed to temperatures around 37°C. before inoculation from when exposed later. With all viruses and hosts yet tested, a pre-inoculation exposure increases susceptibility to infection as measured by the number of infections produced by a given inoculum (56). The effect is similar to that obtained by a pre-inoculation period in darkness, but the response to high temperature occurs more quickly and is greater. Prolonged exposure to 37°C. or periods in darkness damage the host and decrease susceptibility. The precise time of treatment needed to give the maximum increase in susceptibility varies greatly with the age of plants, their past treatment, and with their physiological state at the time they are treated.

By contrast with the pre-inoculation treatment, putting plants at 37°C. after they are inoculated decreases the number of infections obtained, but the size of the decrease differs strikingly with different viruses. With some, such as TMV, it is only slight but with others, tobacco necrosis and tobacco ringspot, for example, infection is entirely prevented. The viruses that fail to become established in inoculated plants placed at 37°C. also fail to maintain themselves when fully (systemically) infected plants are kept for long periods at this temperature, and it is against diseases caused by these viruses that heat therapy is now widely used to get virus-free clones from vegetatively propagated varieties, all the stocks of which have become infected (57). As

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these viruses are inactivated at 37°C. in cells where they were already fully established, their failure to infect at this temperature requires no further explanation. Also, as they are viruses with a small temperature coefficient of heat inactivation, they may be directly inactivated by the high temperature, but as they are more stable in sap than *in vivo* at 37°C. (56), some host system may play a part in their inactivation.

TMV and other viruses with elongated particles have a large temperature coefficient of heat inactivation, but TMV nucleic acid has a small one (58). That the inactivation of tomato bushy stunt virus *in vivo* at 37°C. primarily involves the nucleic acid is indicated by the fact that, although the infectivity of sap from systemically infected plants placed at this temperature falls greatly in the first few days, the content of specific antigen (the virus protein) does not (56). Not all strains of one virus behave similarly in plants at 37°C.; for example, most strains of cucumber mosaic and tobacco ringspot do not infect at this temperature, but some do (59, 60). These differences possibly depend on variations in their protein components, whose constitution or manner of union with the nucleic acid may differ in individual strains and protect the nucleic acid to various extents from inactivation, as does the protein of individual strains of TMV protect the nucleic acid from inactivation by ultraviolet radiation (5).

Analogies for the difference in the response of different viruses in inoculated plants placed at 37°C. are provided by their different behaviour *in vitro* when heated or when exposed to inactivating systems present in mitochondria; viruses such as tobacco necrosis, which fail to multiply in plants at 37°C., readily become noninfective without denaturing or losing their serological specificity, whereas viruses such as TMV that multiply at 37°C. do not (29). What is more difficult to explain is the general effect of heat or darkness applied before inoculation in increasing susceptibility to infection by any virus. Both treatments will, of course, produce many changes in the composition of the leaves. The dry matter and protein content decrease in darkness, while the water and nitrate contents increase (61); ascorbic acid and several other organic acids decrease in amount while citric acid increases (62, 63). If, however, such changes as these are responsible for the increase in susceptibility, they probably act unspecifically by altering the physical properties of the cells, perhaps the osmotic pressure (64), because other methods of changing the leaf content of nitrate (61) and of ascorbic or citric acid (62, 63), have no effects on susceptibility to infection.

From our picture of infection necessitating the separation of nucleic acid from the protein of virus particles and our knowledge of the instability of separated TMV nucleic acid, it is reasonable to seek causes of general changes in susceptibility to viruses in changes of materials that inactivate the nucleic acid. Ribonuclease is one obvious suspect, and the mitochondrial system that inactivates tobacco necrosis and some other spherical viruses (29) is another, for this also inactivates TMV nucleic acid though not the intact virus. There

is no knowledge about how the amounts of these systems are affected by changes in the environment, but should it be found that they decrease in plants kept in the dark or at 37°C. their role in determining susceptibility to infection will still be unestablished. All changes that occur in the dark and at 37°C. will be correlated with increases in susceptibility, but most will be fortuitous, and establishing cause and effect will demand more than simple correlations. For instance, inhibitors of infection normally present in sap from tobacco, potato and *Physalis floridana* decrease in amount as the susceptibility of plants to infection is increased by keeping them at high temperatures (65), but so, too, probably do all other proteins, whether inhibitors of infection or not. Inhibitors of infection occur in many plants (33); they range from proteins to tannins and whether they play any part in determining the susceptibility to infection by viruses of the plants in which they occur is uncertain. Most do not inactivate viruses *in vitro*, but in some way they prevent infection from occurring when they are present in inocula or are rubbed over leaves soon after inoculation with a virus. They more effectively inhibit infection of other plants than of those in which they occur; this fact is not evidence that they have no action against viruses in the hosts which do contain them, but if they have any it seems odd that they should be least effective where they occur in the greatest amounts. Increasing the susceptibility to infection by putting plants in the dark has no effect on the virus-inhibiting power of sap (51). Perhaps the best evidence that some photosynthetic product is concerned in the increase in susceptibility produced by a period in the dark comes from the observation that plants in full light have their susceptibility increased when the air is freed from carbon dioxide, a treatment that has no extra effect when applied to plants kept in the dark (62). Increasing the concentration of carbon dioxide from 30 to 60 per cent in the air in which plants are kept immediately before or after inoculation, greatly decreases susceptibility (66), but possibly by affecting a quite different cell system from that concerned when illumination or temperature is altered.

The gross effects of heat and light (or, rather, darkness) on susceptibility of plants to infection come from treatments that plants would not normally encounter, but less extreme conditions also probably have some effects, and the reported diurnal variations in susceptibility to infection (67, 68) presumably result from the interacting effects of changes in light and temperature during a normal day. Changes in temperature affect more than the susceptibility to infection, for, as with other biological processes, all phases of virus activity are temperature-dependent. Increasing the temperature at which infected plants are kept up to an optimum, which differs with different viruses and even with one virus in different hosts (69, 70, 71, 72, 73), increases the speed at which symptoms appear, their severity, the rate at which viruses multiply and accumulate, and the time taken for them to pass from the epidermis of inoculated leaves to deeper tissues (74, 75). Not all

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processes, however, have the same optimal temperature; a tobacco necrosis virus, for example, multiplies and moves from the epidermis quicker at 30°C. than at 22°C. but it accumulates more rapidly at 22°C. than at 30°C. (74). At 30°C. this virus multiplies and is inactivated simultaneously; both processes probably also proceed at lower temperatures, but this is not easy to demonstrate. At lower temperatures inactivation is relatively unimportant and the rate at which the virus accumulates is possibly a reasonable measure of its rate of multiplication, but inactivation becomes increasingly important as the temperature increases above 22°C.; above 30°C. it becomes the dominant process. Between 22° and 30°C. increasing the temperature may also increase the rate of multiplication, for its shortens the time taken for symptoms to appear, but because of the increasing rate of inactivation it decreases the rate at which the virus accumulates.

With most virus-host combinations that have been studied, symptoms are most severe at the optimal temperature for virus accumulation. There is, though, no general correlation between the extent to which different viruses accumulate and the severity of the symptoms they cause. Only those viruses that are exceptionally stable *in vitro* also reach and maintain high concentrations *in vivo*, and these are by no means always the most virulent. It seems usual, even at moderate temperatures, for most viruses soon to be inactivated *in vivo*, and those that maintain a steady moderate concentration probably do so because synthesis is continually replacing virus that becomes inactivated. Many do not maintain a steady concentration, but after reaching a high concentration in recently infected plants they decline in amount and the symptoms evinced become less severe. This could mean that only leaves that have developed to maturity before they become infected are physiologically suited to supporting a high rate of virus multiplication or that infection increases the amount of virus inactivators in leaves. Some support for the second interpretation comes from the fact that tobacco plants infected with tobacco ringspot virus, which does decline in amount, seem to contain more of a virus-inactivating system than uninfected plants (29). When leaves "recovered" from tobacco ringspot are freed from infection by exposure to high temperatures, they behave on reinfection in the same way as leaves that are infected for the first time (76). Similarly, the initial concentration in plants infected with dodder latent mosaic virus can be restored by infecting recovered plants with TMV or certain other viruses (77), but whether this happens because the second virus stimulates the synthesis of dodder latent mosaic virus or destroys some host system that previously was inactivating it, is unknown. The mechanism whereby simultaneous infection with potato virus Y increases the concentration achieved by potato virus X (78) likewise lacks an explanation.

Different strains of a virus sometimes have different optimal temperatures for multiplication, and changing the conditions under which a culture is maintained can change it qualitatively as well as quantitatively. In TMV it seems

that avirulence towards tobacco may be linked with ability to multiply readily at high temperatures, and putting inoculated plants at 37°C. regularly produces or selects variants that cause less severe symptoms than the type strain (79 to 82).

Whether the ability of analogues of purines and pyrimidines, such as thiouracil (83, 84) and azaguanine (85), to inhibit virus multiplication in infected plants should be included in a section on host-plant metabolism is debatable, for their action has a simple explanation in the fact that these substances become incorporated in virus nucleic acids (46, 47). My reason for including them is that this explanation does not seem wholly adequate, for not only is there little evidence that particles of TMV containing these analogues are noninfective, but their action on virus multiplication depends greatly on the physiological state of the host to which they are applied. TMV, for example, multiplies more readily and reaches higher concentrations in detached leaves floated in solutions of sugar and phosphate and kept in the light than in leaves floated in water and kept dark (86), but thiouracil has little effect on the amount of virus produced in leaves floated in water and kept dark, whereas it almost stops multiplication in illuminated leaves floated in nutrient solutions (87). This could mean no more than that leaves in water and kept dark contain more uracil than the others, for the inhibiting action of thiouracil on virus multiplication is counteracted by uracil (88), but the failure of the virus to increase normally in the presence of thiouracil cannot be attributed solely to the first-formed particles being "sterile," because immediately the thiouracil is removed, or uracil added, the small amount formed in its presence proceeds to multiply as usual. Also, the fact that thiouracil will prevent further multiplication when applied to leaves some days after they are inoculated and when they already contain much infective virus, suggests that some other factor than "sterility" is involved.

As uracil and only uracil counteracts the action of thiocytosine and thiothymine as well as of thiouracil, it seems likely that some uracil-demanding host system concerned in nucleic acid or protein synthesis or both is affected. That the host is affected by thiouracil is clearly shown by the adverse effects it produces on young leaves, but the relevance of this to virus multiplication is uncertain. Supplying uracil to tobacco, for example, does not prevent thiouracil from causing its usual effects on young leaves, but it allows TMV to multiply; also, thiouracil produces adverse effects in beans, in which it does not prevent the multiplication of a tobacco necrosis virus as it does in tobacco (87). This differential effect against the same virus in different hosts again suggests action through a host system, but this suggestion can be offset by the fact that in one host there is a differential effect against different viruses. In tobacco, as already indicated, thiouracil can be a most effective inhibitor of TMV multiplication, whereas it has little effect against cucumber mosaic virus (89); by contrast, azaguanine has little effect against

TMV but greatly inhibits the multiplication of cucumber mosaic virus. Whether this means that cucumber mosaic virus contains more guanine and less uracil than TMV, or is in some way more dependent on the host's guanine metabolism, is unknown. These analogues of purines and pyrimidines get incorporated into host nucleic acids as well as in those of viruses, and the further study of their action may prove fruitful for a better understanding of both virus multiplication and normal nucleoprotein synthesis. It could also lead to methods of chemotherapy, for although no systemically infected plant has yet been freed from a virus by their use, they do decrease the virus content of some plants, and continued exposure to thiouracil has freed cultures of tobacco tumourous tissue from potato virus Y (90).

THE INFLUENCE OF INFECTION ON HOST METABOLISM

Early work on the effects of TMV infection on respiration gave every possible result; that is, increases, decreases, and no change, were all reported. These seemingly irreconcilable claims are now at least in part explicable. First, infection has only a slight effect and, because infection decreases the water content which detached tobacco leaves attain when kept in conditions of minimum water stress, the effect can depend on whether the results are expressed on the basis of dry weight, or initial or final fresh weights; also, the effect varies with the age of the leaves when they become infected and on the length of time they have been infected when the measurements are made (91, 92, 93, 94). The most striking effect is that, in inoculated leaves, respiration is increased by 10 per cent within an hour after inoculation (91); it continues at this increased rate for some days while the virus content is increasing. It then falls below the respiration rate of uninfected leaves, as it also does in systemically infected leaves when they have a high virus content, perhaps because the virus in such conditions represents about 10 per cent of the dry weight of the leaves that is divorced from respiration (93).

The almost immediate effect of increasing the respiration rate of tobacco leaves by inoculating with TMV is unique among the virus-host combinations yet tested. It does not happen with TMV in *N. glutinosa* (95), or with potato virus X (95) or tobacco etch virus in tobacco (96). These three infections produce greater effects on respiration rates—increases of 20–40 per cent—but these occur only when symptoms appear. With these three virus-host combinations, too, photosynthesis is unaffected until symptoms appear, when it decreases by about 20 per cent, whereas in leaves inoculated with TMV it decreases by about 15 per cent within an hour after inoculation (97). To affect photosynthesis, cells below the epidermis are presumably affected, but in what way is unknown; with the concentrated inocula of TMV used, virus particles may reach the chlorenchyma of tobacco immediately, and the results of experiments with other viruses and hosts, which indicate that

inoculated virus is restricted to the epidermis and that the chlorenchyma becomes infected only after the virus has multiplied in and spread from the epidermis (34, 75), may be irrelevant to this system. The effects on respiration do not necessitate the conclusion that deeper cells are infected directly from the inoculum, for effects on respiration per cell could be much greater than are suggested by measurements on whole leaves; if each cell on becoming invaded has its respiration temporarily increased greatly while its metabolism is being diverted to synthesize virus, after which it falls below normal, then the almost constant rise for some days could reflect this process being continually initiated as successive cells become invaded. Whatever the explanation, the rise in respiration precedes virus formation, for at 20°C. approximately 20 hr. are needed before new virus is produced.

As photosynthesis is decreased and respiration increased by viruses that cause "mosaic" type diseases, their characteristic effect in lowering the carbon/nitrogen ratio of plants is readily explicable. Less easy to explain is the increase in the carbon/nitrogen ratio of leaves with "yellows" type diseases, such as potato leaf roll and sugar beet yellows, for these also increase respiration (98, 99, 100) and decrease photosynthesis (98, 101). The old idea that carbohydrate accumulated in the leaves because the phloem is blocked (102) seems no longer tenable, because leaves of sugar beet plants with yellows lose as much total carbohydrate when plants are in the dark as do uninfected plants (103) and leaf symptoms of potato leaf roll are not correlated with phloem necrosis (104). Infection with yellows virus does not affect leaf number and depresses the dry-matter yield of beet by decreasing both leaf area and net assimilation rate, the two effects being independent of one another (105). It increases the quantity of reducing sugars in the leaf greatly and gives smaller increases in sucrose and starch. The rate of photosynthesis seems not much affected and the great loss of dry matter in the root probably results mainly from increased respiration, especially in the roots. The rise in carbohydrate content of the leaves, with no change in the rate of translocation, possibly results from an increased resistance to the movement of sugars out of the leaf lamina, so that a higher concentration has to be built up in the leaves to allow movement. Potato plants with leaf roll also have a lower net assimilation rate than healthy plants, mainly because photosynthesis is diminished in the rolled leaves; different potato varieties have their net assimilation rate decreased to different extents and the leaf area of some varieties is also decreased by infection. The main decrease in dry weight occurs in the tubers (106).

Many of the external symptoms of virus-infected plants, the leaf deformities, witch's brooms and the like, suggest disturbances in the functioning of hormones. The amount of free auxins has been found to be decreased by infecting various plants with a range of different viruses (107, 108, 109). Applying auxins to infected plants usually enhances symptoms rather than counteracting them, but gibberellic acid overcomes the stunting of tobacco

plants caused by etch virus (110), and of asters, corn, and clover infected respectively with aster yellows, corn stunt, and wound tumour viruses (111). Other signs of virus infection are retained, and after two months the growth of treated tobacco plants infected with etch virus falls to that of untreated plants.

Treatments that stimulate rapid stem elongation may have practical applications in helping to obtain virus-free plants from infected stocks. The physiology of meristematic cells seems to be such that it either precludes virus infection or, at most, supports virus multiplication poorly. Many viruses do not penetrate into apical meristems, or, if they penetrate, they fail to survive there. Virus-free plants can be regenerated from plants infected with such viruses by excising their apical stem meristems and culturing them *in vitro* (112, 113). Even viruses that reach apical meristems, or cells so close to them that viable meristems able to grow into plants cannot be excised virus-free, seem to occur in these regions at lower concentrations than in fully differentiated tissues (114). By treating plants with gibberellic acid or other substances that make stems elongate rapidly, the meristems could perhaps be removed far enough from infected cells to allow them to be excised virus-free and viable, and produce a comparable result to that already achieved with carnations, from which viable, virus-free meristems have been excised after decreasing the virus content of plants by keeping them for some weeks at 40°C. (115).

The failure of most viruses to be seed-transmitted, although there seems to be nothing in embryos that specifically inhibits or inactivates viruses (116), may also reflect the inability of viruses to become established and alter the metabolism of dividing cells. The failure to infect probably occurs at or before the time of the reduction division. Nucleic acid and nucleoprotein synthesis will be unusually active in dividing cells and support for the idea that such intense activity may prevent plant viruses from becoming established and diverting the host's mechanisms into the new directions they take in less active cells, comes from the fact that, while recovering in visible light from the effects of exposure to ultraviolet radiation, presumably while repairing damage to nucleic acids, leaf cells also resist infection by viruses to which they are normally susceptible (30).

Ideally an article such as this would end with a section that relates virus multiplication to symptom production, but unfortunately knowledge is still far too meagre to permit this. An outline sketch can now be drawn of some of the events concerned in the establishment of infection and the production of new virus, but few of the details can be filled in with certainty. Even so, information on these events is greater, and accrues more rapidly, than on the ways in which viruses cause the diseases that make them economically important. A better understanding, either of the factors that determine the successful establishment of infection or of the mechanisms underlying the production of symptoms, may have to wait on the identification of the cell

systems that viruses invade. The physiological studies on diseased plants might have been expected to indicate the most likely systems, but they give no clear lead. Indeed, they rather suggest that, if there is one specific type of system that is necessarily invaded as a part of the infection process, then viruses have secondary effects on other systems, because not only are the usual mechanisms for controlling growth impaired, but many viruses affect both respiration and photosynthesis, activities that are probably independent of one another and proceed at different sites in leaf cells. This knowledge strengthens the thesis that virus diseases represent comprehensive changes in host metabolism, but it leads to no further understanding of the intimate interactions between virus and host other than the rather diffuse concept of infection adding new controlling characters to the cell. This it may do, perhaps, by the fusion or association of genetic material from virus and host, to produce a result for which the simplest, but possibly wholly inadequate, analogy is the changing of one variety (genotype) of plant to another.

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PLANT CHEMOTHERAPY[1]

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INTRODUCTION

The subject of plant chemotherapy has expanded greatly in the last few years. There are two reasons for this. The subject is appealing to the plant pathologist because, in principle, he can bring under control groups of diseases that formerly could be combated only by indirect methods. Also, a number of compounds are now known to be effective under field conditions and these, both antibiotics and synthetic compounds, are readily available for experimental and even for commercial use.

Plant chemotherapy has been defined as "the control of plant disease by compounds that, through their effect upon the host or pathogen, reduce or nullify the effect of the pathogen after it has entered the plant" [Dimond *et al.* (1)]. To be effective the compound must be inside the plant where it may (*a*) kill the pathogen as it enters the host, (*b*) rid the host of an established pathogen, or (*c*) mitigate disease [Horsfall & Dimond (2)]. A so-called "systemic fungicide" is a chemotherapeutant but not vice versa. A systemic fungicide fulfills functions (*a*) and (*b*) above, but not (*c*). Compounds applied to plant surfaces may be both protectants and chemotherapeutants; i.e., a portion of the spray residue remains on the surface of the host where it kills the pathogen before it enters, and another portion of the compound enters the host and negates the action of the pathogen from within [Natti (3), Taylor (4), Zaumeyer (5)].

The present review will be concerned primarily with the physiological aspects of plant chemotherapy. Thus, after illustrating plant chemotherapy with a few examples of the control of different types of pathogens, we shall discuss the modes of action of chemotherapeutants, the development of resistance by pathogens to chemotherapeutic agents and the relation of fungitoxicity to activity. Then properties affecting performance will be considered, including availability to biological systems and stability. Entry into the host and translocation of chemotherapeutants will then be discussed. Finally we shall consider natural chemotherapy in two categories: chemotherapeutants synthesized in the environment and those synthesized by the host itself. A more detailed summary of the use of specific compounds in combating diseases has been prepared [Dimond (6)].

The concepts of plant chemotherapy and the use of synthetic compounds for this purpose have been reviewed previously (2, 7 to 14). Other reviews deal with the use of antibiotics in plant disease control (5, 15 to 18).

[1] The survey of literature pertaining to this review was concluded April, 1958.

ILLUSTRATIONS OF SUCCESSFUL CHEMOTHERAPEUTANTS

Before proceeding to a discussion of the physiology of chemotherapy, we shall present a few examples of success in developing the field. For various reasons some of these are unlikely to be adopted commercially.

The availability of effective antibacterial antibiotics is perhaps the reason why bacterial diseases have received so much attention in chemotherapy studies. The use of streptomycin in sprays to combat fireblight of apple and pear, long a scourge to fruit growers, has caused widespread interest among plant pathologists. Disease control in the field has been reported by Ark (19), Winter & Young (20), Dunegan *et al.* (21), Goodman (22, 23), Goodman & Hemphill (24), Hemphill & Goodman (25), Clayton (26), Kienholz (27), and Miller (28). As high as 84 per cent control of this disease has been reported in field experiments and the widespread and growing interest of plant pathologists is evidenced by the great increase in number of papers dealing with this phase of plant disease control.

Virus diseases have also been reduced in severity by chemotherapy. Here evaluation is complicated by the fact that results are sometimes expressed in terms of the suppression of symptoms, rather than in terms of the quantity of virus in the plant with, or without, treatment.

Both antimycin and malonic acid, specific inhibitors of respiration, reduced yields of tobacco mosaic virus in direct proportion to their effect on respiration [Ackermann (29)]. Malonic acid consistently decreased the content of tobacco mosaic virus and fumaric acid overcame the effect of malonic acid in studies of Ryzkhov & Marchenko (30).

Synthesis of tobacco mosaic virus was decreased when inoculated tobacco leaf discs were supplied with solutions of thiouracil. This effect was overcome in part by the addition of uracil to the solution. These results led to the suggestion that thiouracil blocks the synthesis of tobacco mosaic virus by blocking uracil metabolism [Commoner & Mercer (31, 32)]. Nichols (33, 34) has confirmed these results. When thiouracil replaced uracil to the extent of 10 to 12 per cent in the ribonucleic acid moiety of tobacco mosaic virus, the concentration of infective particles was considerably less than that from normal virus. Thiouracil, while unable to inhibit multiplication of all the units of a virus particle, reduces the proportion capable of duplication in the host and thus lowers infectivity [Jeener (35)]. The rate at which virus multiplies is decreased by spraying tobacco leaves with solutions of thiouracil. Multiplication of virus can be checked at any time, but treatment is most effective when leaves contain little virus. Multiplication is resumed when thiouracil is removed [Bawden & Kassanis (36)].

A similar picture has developed for the antiviral action of 8-azaguanine [Matthews (37 to 40), Millikan (41)].

When applied as a foliar spray cycloheximide has given reasonable control of the fungus disease, cherry leaf spot [Hamilton & Szkolnik (42)]. Cycloheximide and its acetate are translocated to new growth and protect it against invasion by the cherry leaf spot pathogen for two to three weeks. The acetate and carbazone were systemically effective at 10 p.p.m., protective at

0.5 p.p.m., and eradicative at 4 p.p.m. [Hamilton, Szkolnik & Sondheimer (43)]. Cycloheximide semicarbazone protected tissue inoculated two weeks after treatment, including tissue developed after treatment was made [Hamilton & Szkolnik (44)].

Geranium plants seriously diseased with bacterial blackleg and *Fusarium* root rot, have been cured by using a mixture of 8-quinolinol sulfate and streptomycin as a soil drench [Stoddard (45)]. Both on an experimental basis and in commercial greenhouses, worthlessly diseased plants have been cured and made commercially salable.

A start has been made in chemotherapy against nematodes. Soil treatments with sodium selenate were found to bring foliar nematode of chrysanthemum under control [Dimock (46)]. Meadow nematodes of the genus *Pratylenchus* attacking boxwood were also reduced by the application of sodium selenate to the soil [Tarjan (47)]. Parathion is also useful for this purpose [Tarjan (48)]. Tomato roots already infested with the root knot nematode, *Meloidogyne incognita*, grew normally when treated with 3-*p*-chlorophenyl-5-methyl rhodanine applied as a soil drench. Root knots failed to develop. Although directly toxic to nematodes, this compound acted in a chemotherapeutic manner by ridding infested plants of the nematodes and preventing root knot formation [Tarjan (49)].

MODES OF CHEMOTHERAPEUTIC ACTION

The Three Modes

Three mechanisms have been proposed to account for plant chemotherapy: that the compound acts directly on the pathogen, that it neutralizes a toxin produced by the pathogen, or that it acts on the host plant to increase its resistance to disease [Horsfall & Dimond (2), Horsfall (11), Dimond *et al.* (1)]. The term systemic fungicide implies a material which is translocated freely in the plant and active directly against the pathogen. Unfortunately it has been used synonymously with the term chemotherapeutant. The latter is the broader term employed to describe any compound having any mode of action to alleviate disease within the plant. Three types of chemotherapy can be distinguished: topical chemotherapy, systemic chemotherapy of local infections, and systemic chemotherapy of systemic infections [Horsfall & Dimond (2, 8), Horsfall (11)].

Killing the Pathogen Directly

An example of a compound that acts directly against fungi within the plant, i.e., a systemic fungicide, is 4-nitrosopyrazole. When the bottom leaf of a tomato plant was immersed in a solution, enough of this highly fungitoxic compound was absorbed and distributed in thirty-six hours to reduce infection of *Alternaria solani* to 44 per cent of that in untreated plants [McNew & Sundholm (50)]. Another example is the fungicide 2-pyridinethiol-1-oxide, which rapidly enters the primary leaf of cucumber and is translocated. The compound is slowly broken down in the plant, however [Sander & Allison (51)].

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Iron, manganese, and zinc salts of this compound and the disulfide gave consistently effective control of *Phytophthora parasitica* var. *nicotianae* on tobacco. Leaf sap contained the disulfide and manganese salts according to Morgan (52).

That most of the antibiotics act directly against the pathogen within the plant is well proven. The antibiotics are directly detectable in the plant by chemical methods as well as by bioassays, and are toxic against pathogens. Thus Pramer, Robison & Starkey (53) introduced streptomycin into chrysanthemum cuttings and used two strains of the bacterial wilt pathogen, *Erwinia chrysanthemi*, as inoculum. One strain was highly resistant to streptomycin and the other was susceptible. Half of the treated and half of the control cuttings were inoculated with each strain of the bacterium. In another experiment, cuttings were rooted in sand containing 50 p.p.m. of streptomycin or in untreated sand. In each case, the antibiotic-resistant strain infected both treated and control cuttings, whereas the antibiotic-susceptible strain infected only the control cuttings.

Development of resistance by the pathogen.—Development of resistance by insects to insecticides and by human bacterial pathogens to antibiotics is well known. Plant pathogens also can develop resistance to chemotherapeutic agents. As yet, examples of this are limited to bacteria developing resistance to antibiotics. Thus *Xanthomonas phaseoli*, the cause of common blight on beans, has developed strains that will tolerate concentrations of streptomycin as high as 0.1 per cent, whereas *Pseudomonas phaseolicola*, the cause of halo blight of beans, does not develop resistant strains nearly so readily [Mitchell, Zaumeyer & Anderson (54)]. *Xanthomonas phaseoli* var. *fuscans* and *X. vignicola*, two plant pathogenic bacteria, after serial transfer on media containing the antibiotic A-6, built up resistance to concentrations of this antibiotic from 130 to 400 times the initial lethal concentration [Carmona-Gomez (55)]. Development of resistance by bacteria has been found to be retarded when combinations of antibiotics were used. Although *Erwinia amylovora* and *Xanthomonas vesicatoria* rapidly build up resistance to streptomycin on serial transfer, the build-up of resistance was very much slower when combinations of streptomycin and oxytetracycline (terramycin) were used [English & Van Halsema (56)].

The build-up of resistance to a chemotherapeutic agent is thus a hazard of plant chemotherapy. Observations of this are so far limited to bacteria in response to antibiotics. How serious a limitation this will be upon chemotherapy of plant diseases in the field, it is difficult to say at the present time. The fact that very few fungi have developed a resistance to protective fungicides in the field offers an optimistic hope that the development of resistance will not present a serious obstacle to success in plant chemotherapy.

Relationship between fungitoxicity and activity.—In our own researches on chemotherapeutic activity for *Fusarium* wilt of tomato, we found more and more examples of effective compounds that were not toxic to the causal fungus [Davis and Dimond (57), Dimond *et al.* (1)]. Even within related groups of compounds no correlation seemed to exist between these variables [Fawcett, Spencer & Wain (58, 59), Fawcett, Wain & Wightman (60)].

These results impel one to seek for other modes of chemotherapeutic activity than mere systemic fungicidal action. These are the possibilities of antidoting fungus toxins and of increasing the physiological resistance of the host.

Neutralizing Toxins

Some cases of plant chemotherapy seem to be due to neutralizing toxins produced by the pathogen within the host. Because culture filtrates of the pathogen frequently will produce toxic effects upon a host, the role of toxins in pathogenesis has often been stressed, yet the number of cases is still small in which a known toxic compound is produced by a pathogen *in vivo*. The term vivotoxin has been proposed by Dimond & Waggoner (61) to distinguish between a toxic material that produces damage in the diseased host and one that is present in a culture filtrate of the pathogen, but which has not been demonstrated in the diseased plant. Wildfire of tobacco is a case in point. The pathogen produces a toxin in the host that forms a halo around the infected lesions. This toxin has been characterized and identified by Braun (62) and by Woolley, Pringle & Braun (63).

The haloing symptoms can be overcome by administering methionine to the plant. Treatment in this case is a good example of chemotherapy of a bacterial toxin; even if methionine is too costly for practice, it is still useful in the demonstration of a principle.

Lycomarasmin, on the other hand, is a toxic substance that is produced in cultures of *Fusarium oxysporum* f. *lycopersici*, the cause of tomato wilt [Gaumann (64)]. It has never been recovered from the diseased plant. Lycomarasmin is a chelating agent that is probably not toxic by itself, but forms a material highly toxic to plant tissue when chelated with iron. The copper complex is nontoxic. If lycomarasmin really plays a role in *Fusarium* wilt of tomato, its effect should be antidoted by feeding the plant copper sulfate. Upon making such an experiment, we found that copper sulfate entered the plant, but did not reduce any symptom of the disease. Another chelating agent that is taken up by the plant, 8-quinolinol, will compete for iron with lycomarasmin *in vitro* and render it nontoxic. 8-Quinolinol has no effect on the disease *in vivo* [Waggoner & Dimond (65)]. If these experiments had come out differently, we would have obtained evidence for both the vivotoxicity of lycomarasmin and the means of chemotherapeutically treating at least one symptom of *Fusarium* wilt.

The vivotoxicity of fusaric acid in *Fusarium* wilt has now been demonstrated by Lakshminarayanan & Subramanian (66) and by Kalyanasundaram & Venkata Ram (67). Chemotherapy by neutralizing this toxin is now a distinct possibility.

As other toxins are identified and their chemical structure becomes known, a very rational approach to chemotherapy will be possible. In other diseases, toxins have been postulated and chemotherapeutants have been developed, which are believed to act against the toxin. These instances have been reviewed by Horsfall (11).

Increasing resistance of the host.—A third mode of action of chemo-

therapeutants is to increase the resistance of the plant to the pathogen. Natural disease resistance in plants is sometimes attributable to the presence of a specific and relatively simple compound that can be synthesized in the laboratory. Although protocatechuic acid confers resistance to onion smudge in red onions, it is not useful as a chemotherapeutant when applied to bean plants for the control of bacterial blight [Dimond, Stoddard & Chapman (68)]. A wide variety of compounds has been tested in chemotherapy assays, notably the assay for *Fusarium* wilt of tomato. Among the compounds tested are the growth regulators. These compounds reduce the severity of *Fusarium* wilt with reasonable consistency. For example, 2,4-dichlorophenoxy acetic acid, α-naphthalene acetic acid, and indole acetic acid all behave in this manner when plants are treated prior to inoculation. The levels that tomato plants will tolerate without serious injury are far below that necessary to poison fungi. Experiments employing tissue or sap of treated plants gave no indication of fungitoxicity [Davis & Dimond (69, 70)]. The suppression of disease is generally related to the time between the treatment with the auxin and inoculation with the pathogen. On the other hand, maleic hydrazide, an antiauxin for many plants, increases the susceptibility of the tomato to *Fusarium* wilt and again the magnitude of the response depends upon the timing of treatment in respect to inoculation [Waggoner & Dimond (71)]. Maleic hydrazide also suppresses the development of crown gall, caused by *Agrobacterium tumefaciens* [Waggoner & Dimond (72)]. Apparently, then, the auxins alter the resistance of the host to disease.

Growth hormones are known to alter sugar levels in plants and the pattern varies with the time elapsing after a plant is treated. Horsfall & Dimond (73) have summarized the relation between sugar levels in plants and susceptibility to disease. Boron deficiency affects sugars in plants by reducing their translocation [Mitchell, Dugger & Gauch (74), Gauch & Dugger (75)]. Treatment of plants with maleic hydrazide also reduces sugar translocation. All such treatments reduce sugar levels in some portions of plants and may increase them elsewhere. Certain diseases prefer tissues high in sugar whereas others prefer tissues low in sugar. Analyzed on this basis a consistent pattern was found by Horsfall & Dimond (73). This hypothesis makes understandable the observation that root injury alters susceptibility to disease. Thus injury to tomato roots brought on by hot water, hydrogen peroxide, and a number of root-injuring synthetic chemicals increases their resistance to tomato wilt [Keyworth & Dimond (76)]. Treated plants were higher in soluble carbohydrates and *Fusarium* wilt appears to be a disease preferring low carbohydrate levels in the plant.

PROPERTIES AFFECTING PERFORMANCE OF CHEMOTHERAPEUTANTS

A chemotherapeutant may or may not be available for the work to be done. For example, it may be adsorbed into objects in the environment or it may not be able to permeate the tissue.

Electric Charge and Availability

Negatively charged soil particles, leaves of plants, and the walls of xylem vessels bear a negative electrokinetic charge when moist; this charge alters the behavior of cationic therapeutants on soil or plant surfaces or within the plant.

Thus, streptomycin bears a positive charge and it is so strongly adsorbed onto soil particles that it is unavailable to plants for absorption or to soil microorganisms [Siminoff & Gottleib (77)]. As a consequence, it must be applied to foliage to combat plant diseases. If used as a dust, however, the same problem appears. Streptomycin is useful for pear blight in a pyrophyllite carrier but not in bentonite which adsorbs it too tightly [Ark & Alcorn (78)]. The addition of K_2HPO_4 aids the liberation of streptomycin from betonite. Other talcs and clays likewise were unsuitable carriers of streptomycin dusts but sulfur and calcium or magnesium carbonates readily released streptomycin to cucumbers for the control of angular leafspot [Ark & Wilson (79)].

Both chlortetracycline and oxytetracycline are adsorbed on soils containing illite and bentonite clays, and thus fail to inhibit sensitive organisms in the soil [Martin & Gottlieb (80)]. Circulin, neomycin, subtilin, viomycin and actinomycin are all adsorbed to some extent on soil [Martin & Gottlieb (81)]. Thiolutin is also adsorbed onto particles of soil or sand [Gopalkrishnan & Jump (82)].

The electrokinetic charge also influences a spray deposit on foliage as shown by Rich (83). In a cataphoretic cell, Bordeaux mixture proved to be cationic whereas zinc ethylene bisdithiocarbamate is anionic. The build-up of spray deposit on the negatively charged leaf surface follows an adsorption curve in the case of Bordeaux mixture and a linear curve in the case of zinc ethylene bisdithiocarbamate. Redistribution is efficient for Bordeaux mixture and inefficient for zinc ethylene bisdithiocarbamate. In the case of streptomycin (positively charged), behavior should be similar to that of Bordeaux mixture with the result that spray deposits should be heavier and redistribution of deposits following weathering more favorable to disease control than if the compound bore no charge or a negative one. Thus the build-up, retention, and redistribution of the deposit on foliage is as much a consideration for the chemotherapeutant as for the protectant fungicide. Other things being equal, the more toxicant and the better the distribution on the surface of the plant, the greater will be the amount entering the plant.

Stability.—Many instances are known where a chemotherapeutant has decomposed to an inert material before entering the plant. In the soil, a multitude of opportunities exist for this to happen. Because the detection of antibiotics is reasonably simple, their decomposition in soil has been better studied than in the case of synthetic compounds.

Three causes of decomposition of antibiotics in soils have been noted by Jefferys (84). Antibiotics may be unstable at the pH of a particular soil and

breakdown of albidin, frequentin, gliotoxin, penicillin, and viridin have all been observed to occur in soils under this condition. Even when the pH of a soil is compatible with the stability of a compound, chemical decomposition occurs in soils. This has been noted in the case of gladiolic acid, penicillin, and streptomycin. Microorganisms degrade griseofulvin, mycophenolic acid, and patulin in soil. Thus these antibiotics disappear in nonsterile soils but persist longer in heat sterilized soils (84). Wright & Grove (85) have shown that griseofulvin slowly disappears from soils, but successive additions disappear more rapidly. A *Pseudomonas* sp., which increased steadily in the soil as griseofulvin was successively added, degraded griseofulvin, dechlorogriseofulvin, and the amine salt in culture.

The plant itself can and often does detoxify chemotherapeutants. The decomposition of griseofulvin within the broad bean plant is exponential and the compound has a half life of about four days [Pramer (86), Crowdy (87)]. In cherry fruits cycloheximide has a half life of about 24 hours and there is evidence that an enzyme system in the ripe fruit accelerates inactivation [Prescott, Emerson & Ford (88)]. However, cycloheximide and its derivatives are not broken down so rapidly in cherry foliage. The oxime is absorbed through foliage and translocated to new growth in sufficient amounts to protect it against cherry leaf spot for two to three weeks [Hamilton, Szkolnik & Sondheimer (43)]. Likewise, in certain plants, at least, streptomycin appears to be reasonably stable in plant tissues. Although diluted in concentration by growth of the plant, streptomycin remained in fully developed tobacco leaves for more than five weeks [Hidaka & Murano (89)]. However, addition of macerated peach leaf tissue to streptomycin solutions reduced the titer by an amount greater than dilution or alteration of pH in studies of Dye (90). Griseofulvin and chloramphenicol are active as such in the broad bean plant as shown by chemical determination and bioassay in the work of Crowdy *et al.* (91). Cycloheximide was detectable in leaves of wheat seedlings for as long as five weeks after application and chromatographic evidence indicated it was not altered in the plant to another compound [Wallen & Millar (92)].

Cysteine, because of its highly reactive sulfhydryl group, inactivates such materials as penicillin, penicillic acid, patulin, gliotoxin and streptomycin [Bailey & Cavallito (93)]. The inactivation of antimicrobial agents by metabolites has been reviewed by Gottlieb (94). Thus a toxic chelating agent or chelate complex may be detoxified by chelation with a metal to form a nontoxic complex that is more stable than the original compound. Competitive antagonism results in detoxification when an organism blocks the action of a toxicant by producing a metabolite in quantity. Proximity effects and enzymatic breakdown of the toxicant are other ways in which chemotherapeutants can be inactivated by the plant.

Sometimes a compound applied to the plant for disease control may be converted to a more potent compound. This possibility is well known from work with systemic insecticides. Cycloheximide acetate-2-C^{14} is altered in plant tissues to the more toxic free cycloheximide [Lemin & Magee (95)].

Streptomycin amine and streptomycin oxime are converted in bean or tobacco tissue to a more toxic compound, probably streptomycin or dihydrostreptomycin [Gray (96)].

ENTRY INTO THE HOST AND TRANSLOCATION

Obviously a chemotherapeutant must enter the host plant and be translocated if it is to exert a significant systemic effect. Injection may be used to insert it, but entry through foliage or intact roots seems more satisfactory.

Many plant growth regulators and related compounds act as chemotherapeutants. The absorption and translocation of these compounds has been reviewed by van Overbeek (97). Organic compounds must be adsorbed on the cuticle and then permeate through the plasma membrane into the cell, the second process being temperature-dependent. Relations of balance between solubility of fungitoxicants in lipoidal and aqueous phases and permeative ability into the cell have been analyzed in relation to chemical structure by Horsfall (11).

The ability of *Nitella* cells to absorb chloramphenicol, penicillin, and streptomycin was studied by Pramer (98). Streptomycin was rapidly absorbed and after twelve minutes, the concentration in the cell equalled that in the ambient solution. After eighteen and a half hours the concentration in the cell was seven times that in the ambient solution. Accumulation was temperature-dependent and had an optimum pH of 5.0. Respiratory inhibitors had no effect on the accumulation. The energy of activation of the transport system was approximately 7500 cal./mol. [Litwack & Pramer (99)]. Chloramphenicol appeared much more slowly in the cell and after twenty-four hours was present in a concentration one-half that of the ambient solution. In contrast to the active transport across the membrane shown by streptomycin, the entry of chloramphenicol followed a pattern of simple diffusion. Penicillin could not be detected inside the cell (98).

The uptake of a compound by the plant can be easily measured by bioassay, in the absence of a reliable micromethod specific for the compound. In studies involving antibiotics, the ability of treated plant tissue to produce a zone of inhibition on nutrient agar seeded with a sensitive bacterium has frequently been used. Streptomycin-dependent strains of bacteria are very useful in detecting the presence of streptomycin in plant tissue.

Less refined methods have also been used. King (100) has dipped leaf tips of corn seedlings in the test solution. Uptake and movement have been inferred from the pattern and symptoms of phytotoxicity appearing at a distance from the point of uptake. The water balance of the test plants determines the type of result obtained with this method. Another method, developed by Van Raalte (101), has been employed for studies on systemic fungicides. Compounds toxic *in vitro* to the fungus *Penicillium italicum* are placed on a cylinder of potato petiole about 5 mm. long. The other end of the cylinder is placed on nutrient agar seeded with the test fungus. A zone of inhibition in growth of the fungus indicates the ability of the compound to

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diffuse through the plant tissues. In limited trials, correlations between performance of materials and their known ability to translocate are good.

Entry into Cut Stems

When injected into trunks of oak trees, cycloheximide, cycloheximide acetate, and oligomycin are translocated in xylem tissue [Phelps, Kuntz & Riker (102)].

Antibiotics are more readily taken up and translocated by stem cuttings than by roots [Robison, Starkey & Davidson (103)]. This would be expected because there is no cuticular barrier in cuttings and movement is with the transpiration stream. However, even in cuttings, antibiotics vary in translocatability. Thus in *Pyracantha* and carnation cuttings tetracycline, chlortetracycline and oxytetracycline moved from the base to the apex more rapidly than streptomycin or neomycin. Streptomycin moved more rapidly upon the addition of 1 per cent K_2HPO_4 [Alcorn & Ark (104)]. The strong tendency of streptomycin to be adsorbed on negatively-charged surfaces such as vessel walls probably accounts for this.

Entry Through Roots

Anderson & Nienow (105) demonstrated the entry of streptomycin into roots of soybean plants and its translocation to leaves. The many experiments on entrance and movement of antibiotics in plants have been reviewed by Crowdy & Pramer (106) and by Brian (107).

The uptake of the same antibiotic differs from one plant to another. In peach seedlings, roots absorb streptomycin and the concentration in leaves rises above that in roots. In upper leaves the concentration is less than in lower leaves [Dye (90)]. On the other hand, in cucumber, streptomycin and chloramphenicol concentrations in the plant remain lower than the ambient fluid, whereas chlortetracycline, oxytetracycline and neomycin are not absorbed [Pramer (86)].

In the broad bean plant, roots at first contain more chloramphenicol than tops, but later the concentration in the plant becomes uniform. Tomato plants absorb streptomycin more rapidly than broad bean plants do [Pramer (108)].

In tomato and broad bean, streptomycin is less mobile than chloramphenicol. In the broad bean streptomycin tended to remain in roots whereas in the tomato, some moved up to leaves. The concentration of streptomycin in the top of the plant increased with increasing concentration of solution supplied the roots. At low concentrations, streptomycin appeared in the top of the plant only after a time lag, which suggests that root tissues must become saturated with the compound before it can be moved to the top. There was no such lag in the case of chloramphenicol or of griseofulvin. Evidently root tissues have no special affinity for the latter two compounds.

On removing chloramphenicol-treated plants to water, the antibiotic moved from the roots to the top and from lower to upper leaves. In beans, the antibiotic, griseofulvin, left the roots completely, and gradually dis-

appeared from the stems. Tomatoes did not redistribute these antibiotics.

After a long absorption period, griseofulvin in beans is found at highest concentration in the lower leaves, lower concentrations in the upper leaves, little in the roots and very little indeed in the stem. In tomatoes the roots contain most, the lower leaves less, and the upper leaves still less; however, the upper leaves contain much more than the stems [Crowdy (87)]. Although cycloheximide was readily absorbed and translocated by wheat plants and moved throughout stems and leaves from roots, it was not detected in tissues that grew after the application [Wallen & Millar (92)].

Some antibiotics seem to move in the xylem. For example, griseofulvin is exuded in the guttation drops of wheat plants when it is absorbed through roots [Stokes (109), Brian *et al.* (110)]. The time before appearance in guttation fluid varied from two days in summer to seven days in winter [Brian (9)]. Cycloheximide also appears in guttation fluid of wheat plants when roots are immersed in the antibiotic [Wallen & Millar (92)]. Thiolutin does not appear in guttation droplets of treated tomato plants [Gopalkrishnan & Jump (82)].

Other evidence for movement in the xylem is the fact that conditions favoring transpiration increase uptake and translocation of griseofulvin [Stokes (109)]. The rate of chloramphenicol uptake is also proportional to the rate of water uptake [Pramer (108)]. The uptake of sulfonamides is conditioned by the transpiration stream [Crowdy & Rudd Jones (111)].

The respiratory inhibitors sodium azide and phenyl urethane inhibit uptake and translocation of griseofulvin in wheat plants [Stokes (109), Brian *et al.* (110)]. Thus the absorption of griseofulvin by broad bean follows a pattern. Other conditions being equal, the rate of uptake is proportional to the concentration of antibiotic offered roots below phytotoxic levels. For any given concentration, uptake is proportional to the volume of water transpired. The material decays exponentially in the plant. At any time the concentration in a tissue is the resultant of exponential decay and the linear increase related to transpiration. The initial rapid entry into roots is inhibited by sodium azide and nitrophenol at concentrations that do not reduce transpiration. The prolonged uptake is linearly related to transpiration and is not affected by these inhibitors [Crowdy *et al.* (112)].

The uptake and movement of sulfonamides in the broad bean plant is similar. Crowdy & Rudd Jones (111), using chemical methods for detecting a series of sulfonamides, determined that some are absorbed and move readily and others are not. Two types of entry were detected: (*a*) an initial rapid entry that is azide sensitive and (*b*) a more prolonged entry, not azide sensitive, in which uptake is conditioned by the transpiration rate. No evidence was obtained for an active phase of uptake. According to these same investigators (113), an analogy exists between the uptake of sulfonamides by roots and their partitioning on cellulose chromatograms in which the movement across the cortex to the vascular system and their Rf value on a chromatogram are both attributed to a partitioning mechanism. Before tissues are saturated with the compound, the rate of accumulation in shoots measures the concentration in the transpiration stream. For equal ambient

concentrations of compounds about the roots, the ratio of the rate of accumulation in the shoot to the Rf on cellulose is constant when conditions for partitioning are comparable. For water and neutral compounds that are lipoid soluble, this ratio is constant. Compounds more soluble in water require a higher proportion of aqueous phase in the solvent system to attain this constant ratio. The p*Ka* values of the compounds exert a critical effect upon the behavior of the compound with respect to entry. Neutral compounds and those with a high p*Ka* value may move in the plant as undissociated molecules, whereas those with a low p*Ka* value may move in the plant as an ion. When the p*Ka* value is in the vicinity of seven, slight variations in the pH may cause a critical shift in the proportion of undissociated molecules. Most, if not all, movement through roots is of a physical nature and not attributable to biological processes.

Entry into Intact Stems

Streptomycin in lanolin with Tween 20 is absorbed when applied to intact stems of bean plants and antibiotic activity appears in leaves [Mitchell, Zaumeyer & Anderson (54), Mitchell, Zaumeyer & Preston (114)]. The antibiotic appeared in primary leaves within eight hours of treatment and concentration was greatest on the third day. Translocation occurred from the tip to the base of the primary leaf, from the midrib to the margin, and from the first internode to the primary and the first trifoliate leaf. There was no downward movement in the stem from the point of application or from primary leaves to the stem or from the terminal to the basal portion of the primary leaf or from trifoliate leaves into the pod [Mitchell, Zaumeyer & Preston (115)]. In tobacco, streptomycin applied to stems moved through phloem and pith, slowly moved upward to higher leaves, but never appeared in xylem [Hidaka & Murano (89)].

Uptake of Antibiotics by Leaves

When applied as a spray to tomato leaves, griseofulvin, applied to the one surface only, entered sufficiently to protect them for as long as seven days against *Alternaria*, inoculated only on the other surface [Davis & Rothrock (116)].

Cycloheximide and oxytetracycline, sprayed on leaves of sweet corn, protected them against infection by the Stewart's wilt pathogen 24 hours later [Williams & Lockwood (117)]. Although streptomycin was not detectable in pepper leaves when sprayed on them, it was found in leaves of snapbean after being sprayed on them [Crossan & Krupka (118)]. In peach seedlings, streptomycin sulfate, applied to lower leaf surfaces, protected them when inoculated on the upper surface with *Pseudomonas syringae*, but there is no evidence that the antibiotic becomes systemic in the plant under these circumstances. There was no movement from the base to the apex of leaves and streptomycin may become bound in tissues after its entry [Dye (119)].

Some climatic factors alter the entry of these compounds into leaves.

Light has no influence on this process of entry of streptomycin into the tobacco plant [Hidaka & Murano (120)].

A high relative humidity aided the entry of streptomycin into leaves of peach seedlings, presumably because the sprayed leaf remained wet longer [Dye (119)]. Thus, the addition to spray formulations of materials that cause the spray drops to remain moist for longer periods (humectants) sometimes improves the entry of compounds into plants. For example, the addition of glycerol to sprays containing streptomycin caused a marked increase in effectiveness against the common bacterial blight of Pinto bean [Gray (121)]. It also improved the entry of streptomycin into leaves of bean, tomato, pepper, and tobacco. The increase in uptake was evident within three hours of spraying, was five times as great after six hours and 24 times as great after 96 hours. Glycerol also increased the entrance of chloramphenicol and streptothricin, but it did not affect the entry of oxytetracycline or of neomycin [Gray (122)]. Sorbitol, diethylene glycol, and other polyhydric alcohols, while improving the entry, were not as effective as glycerol.

The addition of a wetting agent tends to offset the improvement in absorption caused by glycerol because spray drops are smaller and they dry more rapidly than drops of larger size. However, a wetting agent (Tween 20) increased threefold the uptake of streptomycin by *Sedum* leaves according to Lockwood (123). The addition of methyl cellosolve and of Carbowax 4000 has increased the absorption of streptomycin and of oxytetracycline when used for control of fireblight of apple and pears [Goodman (22)]. The improved action of these antibiotics in the presence of humectants was demonstrated in bioassay by Hemphill & Goodman (25).

Addition of glycerol has not improved disease control in all cases when streptomycin was employed. Thus, in control of wildfire on Burley tobacco, the addition of glycerol had slight if any effect [Shaw, Lucas & Thorne (124)]. Likewise glycerol did not improve the activity of streptomycin or of Captan in the control of cucumber scab although they alone effected reasonable control [Rich (125)].

Translocation Resulting from Entry of Materials into Leaves

Applied as a spray to primary leaves of dwarf bean plants, streptomycin sulfate showed a systemic action against the halo blight bacterium as far away as the fourth trifoliate leaf and the action persisted for as long as 11 days [Napier *et al.* (126)]. In tobacco plants, streptomycin on leaves showed a maximum activity eight hour after spraying [Hidaka & Murano (120)]. Applied as sprays to intermediate leaves of bean or tobacco plants, streptothricin or pleocidin were translocated downward to older leaves, and upwards also, but streptomycin, dihydrostreptomycin, neomycin, oxymycin, bacitracin and actinomycin failed to move out of sprayed leaves. However, in some cases these materials moved both upwards and downwards from a leaf immersed in a solution of the antibiotic. Streptothricin and pleocidin were translocated downward to roots as well as upward to younger leaves and when the leaf was immersed in the antibiotics, they were translocated

through steam-killed sections of petioles and stems to older leaves below the ring, indicating that downward movement was principally in xylem [Gray (96)].

Crowdy & Pramer (106), in summarizing the translocatability of antibiotics, state that the readily translocated antibiotics appear to be either neutral or acidic (griseofulvin, chloramphenicol and penicillin), and anomalous results have been reported for basic compounds (streptomycin and neomycin) and for amphoteric ones (chlortetracycline and oxytetracycline).

NATURAL CHEMOTHERAPY

Chemotherapeutants Synthesized in the Environment

One of the borderline fields of chemotherapy concerns the action of naturally produced antibiotics. Soil-inhabiting fungi and bacteria that produce antibiotics are well known and their role in holding down populations of plant pathogens has long been suspected. When antibiotics are synthesized in the environment in such quantity that they can enter the plant and make it resistant to disease, then natural chemotherapy is involved. Otherwise the problem is simply one of microbial ecology.

Antibiotics are produced by fungi when seeds are inoculated with them as shown by the studies of Wright (127). Thus the antibiotics gliotoxin, frequentin, and gladiolic acid were produced in seed coats and identified chromatographically when appropriately inoculated seeds were sown in soils. Also gliotoxin was found in pea seed coats of seed sown in a soil naturally containing vigorously growing *Trichoderma viride*. When *Trichoderma lignorum* was incorporated into pelleted alfalfa seed, damping off by *Pythium debaryanum* was prevented in studies of Gregory *et al.* (128). Thus there can be little doubt that sufficient antibiotics are produced under experimental conditions to enter the plant and protect it against infection.

It is still an open question as to whether the antibiotics serve to reduce pathogenic organisms in the soil or enter the plant and reduce disease in a chemotherapeutic manner. The control of disease by use of antagonistic organisms has been reviewed by Wood & Tveit (129). Antifungal antibiotic activity was observed in sugar cane soils of Louisiana that were already high in *Pythium* root rot, whereas antibacterial antibiotic activity was high in soils where root rot was not severe, a result that suggests that the antibiotics in this case may be exerting their influence in an ecological manner rather than a chemotherapeutic one [Luke & Connell (130)].

The foregoing discussion on stability of antibiotics suggests that they frequently disappear when added to soils. Generally the production of an antibiotic is higher when organic amendments are made to the soil than when the soil is unamended, and in certain instances, the organic amendment must be of a specific type. Kalyanasundaram (131) has shown that fusaric acid protection requires rather large amendments of organic nitrogen and carbohydrate. Thus chloramphenicol was produced by *Streptomyces venezuelae* on an unamended soil, but the yield was greatly increased on addition of tryptone. Yields were low when green alfalfa hay was added but neither

oat straw nor starch encouraged any antibiotic production. Chloramphenicol is rapidly degraded by microorganisms in unsterilized soil [Gottlieb & Siminoff (132)].

Gliotoxin is produced freely by *T. viride* in soils under appropriate conditions. This antibiotic was detected chromatographically in an acid, nonsterile heath soil supplemented with 2 to 5 per cent dried clover, but not in a normal garden soil unless it were first acidified [Wright (133)]. Gliotoxin is stable only at a low pH and is inactivated in garden soils [Jefferys (84)]. Although greater yields were obtained in autoclaved soils, the antibiotic was produced and was detected in nonsterile heath soils around particles of buried organic matter such as wheat straw [Wright (134)]. When soils poor in gliotoxin production were acidified, the antibiotic was produced but not when heath soils were raised in pH by the addition of lime. Inoculation of the soil was necessary and gliotoxin production varied with soil type [Wright (127)].

Griseofulvin was produced by *Penicillium nigricans* when inoculated into a sterile soil amended with corn meal. But when this inoculum was added to nonsterile soil, no further antibiotic was formed and that initially present in the inoculum slowly disappeared [Brian (9)]. A higher level of amendment was required for griseofulvin production and antagonism by other soil organisms depressed production of griseofulvin more than that of gliotoxin [Wright (135)].

Patulin is decomposed biologically in soils [Jefferys (84)]. Its formation has been observed in sterile, unamended soils, but carbohydrate decomposition is essential for its appearance [Grossbard (136, 137)]. It was not adsorbed on bentonite or illite but remained active in soils containing these clays and inhibited growth of sensitive microorganisms in studies by Gottlieb, Siminoff & Martin (138). Patulin was not found in soils infested with *Aspergillus clavatus* even when different amendments were added. Its presence in soil gave rise to a resistant microflora.

In summary, then, antibiotics are apparently produced in soils to a limited extent. Many hazards in this environment reduce the amount of antibiotics available to plants. Production of antibiotics is severely restricted in cultivated soils, where organic content is low. Thus, while antibiotics may be produced and exert an influence on the levels of pathogenic organisms in soils, the likelihood that antibiotics enter plants and there control diseases in a chemotherapeutic manner is rather remote. However, seeds sown in a soil containing antibiotic-producing microorganisms may grow upon organic matter of the seed and protect it against pathogens for a short time.

Chemotherapeutants Synthesized by the Host Itself

In a few cases, the resistance of plants to disease is attributable to a specific toxic compound that is synthesized by the host. This is another type of natural chemotherapy. One approach to chemotherapy is to offer the plant compounds which are similarly fungitoxic and which are absorbed and moved to the infection court.

Phenols and quinones, for the most part, are the substances suggested as

natural chemotherapeutants. Presumably the quinones, which are more toxic than phenols against many fungi, are derived by oxidation of the phenols [Horsfall (11)]. The presence of protocatechuic acid as a pigment in red varieties of onions acts as a natural therapeutant for onion smudge. The nonpigmented varieties, lacking this compound, are susceptible [Walker, Link & Angell (139)]. By now other instances of the principle are known. A quinone, 2-methoxy-1,4-naphthoquinone, has been isolated from *Impatiens balsamina*, a plant which has very few diseases. This quinone is strongly fungitoxic to *Monilinia fructicola* and confers resistance upon the plant, according to Little, Sproston & Foote (140). This compound is closely related to the agricultural fungicide, phygon, or 2,3-dichloro-1,4-naphthoquinone.

The resistance of potatoes to the scab organism, *Streptomyces scabies*, has likewise been attributed to the oxidation of phenolic fractions in the potato to quinone. The phenolic compounds, chlorogenic acid, caffeic acid, catechol, and tetrahydroxybenzoin were found on auto-oxidation to inhibit the growth *in vitro* of *S. scabies*. These results support the theory that the mechanism of scab resistance in potato tubers involves the enzymatic oxidation of chlorogenic acid to a quinone that is toxic to the scab organism [Schaal & Johnson (141)].

The ferric chloride spot test for phenols has been used to measure scab resistance of potato varieties by Johnson & Schaal (142). There is also a significant correlation between response of periderm and cortical tissues of potato tubers in the ferric chloride test and their resistance to *Verticillium* wilt in the field. Chlorogenic acid occurs in stems, leaves, and roots of potatoes and this compound is probably the chromogen in the ferric chloride test [McLean, Le Tourneau & Guthrie (143)]. When potato tubers are inoculated with *Helminthosporium carbonum*, fungistatic substances are produced, according to Kuc, Ullstrup & Quackenbush (144). From such inoculated tissue, chlorogenic and caffeic acids have been isolated and identified chemically. Both of these compounds are fungistatic to *H. carbonum* [Kuc *et al.* (145)]. Chlorogenic acid was synthesized in potato tuber disks as they were incubated, synthesis increasing with sugar level. Oxygen supply usually limits the synthesis. The chlorogenic acid content of refrigerated tubers is low, but on attaining higher temperatures, tubers begin to synthesize this compound according to Zucker & Levy (146).

Phenolic components in the heartwood of pine appear to be responsible for its resistance to decay. The fungitoxic substances, pinosylvin and its monomethyl ether have been isolated from such tissues by Rennerfeldt (147). Cotton varieties resistant to cotton wilt contain antibiotic materials inhibitory to *Verticillium dahliae*, whereas susceptible varieties lack this inhibitor. However, varieties resistant to *Fusarium vasinfectum* contain nothing inhibitory to *Fusarium* [Kublanobskaia & Brailova (148)].

From resistant rye seedlings 2-(3-)benzoxazolinone was isolated. This compound is toxic to *Fusarium nivale* and to *Sclerotinia trifoliorum* and may be responsible for disease resistance according to Virtanen & Hietala (149).

In some cases resistance may be attributable to a metabolic state in the

host, a subject that has been reviewed by Sempio (150). This view is supported by Scheffer & Gothoskar (151). In tomato plants having single gene resistance for *Fusarium* wilt, resistance was completely overcome when plants were treated with 2,4-dinitrophenol, thiourea, or sodium fluoride. Other respiratory inhibitors had no influence on resistance.

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THE MACRONUTRIENT ELEMENTS[1,2]

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The term "macronutrient" is recent, having come into popular usage since the general acceptance of the term "micronutrient." Therefore, the term "macronutrient element" suggests one of the nine elements needed by plants in larger quantity, namely: nitrogen, phosphorus, sulfur, calcium, magnesium, potassium, carbon, hydrogen, and oxygen. However, only the first six which can be supplied conveniently as fertilizers are ordinarily considered among the macronutrients. In plant physiology, the understanding associated with "nutrient elements" is distinctively separate from the general sense of energy-containing foods. Thus, in speaking of "nutrient elements," the implications are always of the simple mineral constituents necessary for the growth and development of the living plant.

A large and expanding need for the macronutrient elements in contemporary agriculture encourages most of the research effort directed toward them. For the same reason, many other specialists besides plant physiologists maintain similar interests in the macronutrients.

A continuing live interest in the chemical elements required for plant growth stems, of course, from the activities of agriculture in providing our "daily bread." The time has long since passed when agriculture can supply food to the more advanced countries of the world by the simple methods of tradition. As agriculture accommodates more and more into the methods of industrialization, it depends increasingly upon other industrial activities.

Agriculture has now become the most sophisticated of all the arts in adapting things of the natural world to the uses of human society. It calls upon every facet of contemporary science to help in meeting the common problem. It enlists industry to provide the continually increasing requirements for machinery and for additional supplies of water, fertilizers, and toxins to fight the unrelenting war against insects, nematodes, and diseases of plants or animals. Macronutrient elements appear clearly as subjects of exceptional importance if for no other reason than their sheer bulk, which has to be moved to suitable geographic regions for economical plant production.

It is very rare when the chemical elements needed for maximal plant growth are supplied adequately by soils. Enormous tonnages of calcium, magnesium, potassium, nitrogen, phosphorus, and sulfur are mined, proc-

[1] The survey of literature pertaining to this review was concluded in October, 1958.

[2] The following abbreviations will be used: 2,4-D (dichlorophenoxyacetic acid; EDTA-Na (ethylenediaminetetraacetate); THO (tritium).

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essed, and transported in the interest of sustained agricultural production. The three remaining macronutrient elements, carbon, hydrogen, and oxygen, are special cases. Carbon dioxide, as an atmospheric constituent, is provided generously by nature, even though plant growth could be enhanced by supplying greater quantities of carbon as carbon dioxide artificially. There is small probability of attention being directed seriously toward this end until demands for plant products become much more critical than they are now. Water, which supplies hydrogen and at least some oxygen for plant growth, in addition to being the indispensable supporting medium for all living organisms, is often supplied through irrigation. Supplying of irrigation water remains one of the most remarkable feats in transportation engineering from historical times to the present. Today, at a price of one cent per ton, water is captured, stored, and transported hundreds of miles to the point where it is to be used. Stringencies placed upon agriculture are such that a cost of two cents per ton for water could mean ruin to many an individual farmer. Ten cents per ton would surely call an absolute halt to effective irrigation under present day economic circumstances.

Availability of supplies of macronutrients is everyone's concern because such supplies have now become an indispensable part of the foundation of agriculture. On the national scale, possession of cheap sources of good water, limestone, gypsum, potassium salts, rock phosphate, elemental sulfur, and inexpensive power for nitrogen fixation is as important as the possession of good lands. An industrialized economy is needed, however, to provide the engineering, chemical processing, and transportation needed to bring fertilizers from the mines to plants growing in the field, at reasonable cost. In the United States we have passed so far toward agricultural dependence upon the mineral fertilizer industry that without it our present surplus agricultural production would be converted promptly to acute shortages. Our agricultural use of elemental nitrogen, phosphorus, and potassium in the United States in 1957 was respectively, 1.9, 0.5, and 0.8 millions of tons [Williams *et al.* (1)]. Interestingly, nitrogen from synthetic sources exceeded that from organics a hundredfold.

Maintaining continuous supplies of macronutrients involves mining operations. In reality, agricultural activities in themselves can be considered as mining for the macronutrients of the soil. There is an established sense of worry and general public concern over this latter fact. Thus, the expression "mining the soil" carries a connotation of bad agricultural practice and farmers are often accused of being unmindful of their responsibilities to lasting public welfare. A sense for conservation brings the same people to insist upon the replacement of products withdrawn from soils with products from regular mines, as good practice. These inconsistencies in attitudes are not hard to explain because people are accustomed to accept the land as a permanent natural resource, whereas mines obviously are not permanent. The broader geochemical view, however, must include consideration of the future of mines as well as the future of soils. The thing to be recognized now and

guarded against is waste. It is not too early to think of directing research toward the conservative uses of macronutrients so that "best practices" will be recognized as such throughout the gross cycle, including the soil, high grade mineral resources, and the methods of refuse disposal.

Reviewing contemporary research on the macronutrient elements reveals the preponderance of effort having been expended toward seeking information to guide the agriculturist in producing greater crops at lesser cost. This is in keeping with the desires of the conservationist as well as being to the agriculturalist's economic advantage. From the point of view of guarding against waste and eventual implications of its careless indulgence, it is not the farmer who "mines the soil" but rather the city dweller who inconsiderately disposes of macronutrients by burying them or flushing them into the sea, also because it is to his economic advantage to do so. Therefore, it may be predicted that future historians in seeking early trends responsible for a later state of affairs will have to look upon our current societies within city and urban areas as primitively negligent in comparison to positive understandings of an enlightened rural population.

Therefore, it makes good sense to look upon farming as a mining operation which exploits the lowest of all low grade ores for the purpose of providing necessities of life. We might consider, for example, the predicament of a mining engineer who might be asked to recover 20 pounds of phosphorus recently distributed over an acre and plowed into the soil. The mining engineer would not consider the proposition as reasonably solvable by methods of mining because of prohibitive expense. However, the farmer would expect to recover some of the phosphorus in a crop of higher yield and would charge nothing for the recovery.

We are greatly in need of complete and accurate knowledge of the mechanisms by which plants extract macronutrients from soils. Herein lies a great challenge for the plant physiologist. We need first of all to know whether there is a difference between plant species in their abilities to extract nutrients. For example, there is popular acceptance of an idea that some species do extract more efficiently than others. Phosphorus is one of the macronutrients most frequently referred to in this respect. If this is indeed so, it has not been demonstrated by unequivocal experiments. It is to be hoped that someone will be able to demonstrate real differences in phosphate-extracting powers of plants in such a way that specific mechanisms can be understood. Eventually we look forward to having the mechanisms so clarified that plant breeders can attempt arrangements of genetic material of crop plants enabling new hybrids to extract adequate supplies of macronutrients from soils that defy the abilities of contemporary varieties.

During the past three years there have appeared well over a thousand papers touching upon the macronutrients in one way or another. In reviewing the status of contemporary research which would lead to new principles, there are not much more than progress reports. Most of the work has to do with the special needs of specific crops. All kinds of crops are cultivated under

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different soil conditions and the methods of getting the macronutrients into the best places to benefit the growing plants call for special investigation. With crops ranging through rice in anaerobic paddies, dry farmed wheat, and conifers on podsolic soils of northern climates, it is not hard to understand the reasons for a great number of investigators working toward the most efficacious methods for gaining increased production.

Agriculturists are usually faced with a narrow margin between profit and loss and look to fertilization as a means for better assurance of successful operation. However, the use of fertilizers involves further consideration of cost. A simple thing such as an increased allowance for freight tariffs is reflected all the way down the line, causing even closer adjustments of farm management practice, with increased demands for better and more detailed knowledge of optimal needs (in the economic sense) of growing plants for supplements of nutrient elements. Consequently, within the fertilizer industry, considerable effort is being directed toward supplying products having the maximum available plant nutrient for the least weight to be transported. Just as assiduously, therefore, plant physiologists and soil scientists direct attention to the relative usefulness of new and different chemical forms of the macronuturients as to their adequacy in supplying plants' needs for macronutrient elements.

Out of this sizable activity, the reviewers have attempted to gather points suitable for generalizations. Within the wide testing ground represented, everyone follows principles of plant nutrition which have been established previously. One of the newer departures is to be found in the many investigations of leaf feeding which, though an unnatural way to introduce macronutrients (or micronutrients) into plants, has excited much curosity and a few successful applications. On the other hand, one meets many references to long standing practices such as "liming the soil." As illustrative of the complexities confronting research workers attempting to provide firm and useful knowledge, one could refer to a paper which brings together the results of 1000 liming tests made in Sweden alone [Lundblad & Ekman (2)]. This work comprises 383 references and the admonition: "Among the most urgent problems for research is that of the interaction between lime and the major and minor plant nutrients." These and similar efforts may not be regarded as in keeping with the objectives of the scientific pursuit of knowledge. However, they are clear evidence of the paucity of available scientific knowledge needed to meet the contingencies of agriculture. Also, many practical questions which require fundamental answers are brought to the attention of investigators which might have escaped notice otherwise.

Calcium, Magnesium, Potassium

There is hardly any field operation involving supplementation with calcium, magnesium, and potassium which does not reflect immediately considerations of interrelations between these three elements. Calcium is peculiar among the macronutrient nutrients in that a large percentage of the

adsorption complex of soil systems must be occupied by calcium if the average crop plant is to receive adequate calcium from soils. It is generally understood that about 30 per cent of the soil's cation exchange complex should be occupied with sorbed calcium if it is to meet adequately a plant's need for this element. The remaining adsorption spots of the exchange complex of a good agricultural soil contain, besides the macronutrients magnesium and potassium, adsorbed hydrogen, aluminum, sodium, and, in lesser amount, the micronutrient cations. Ordinarily, we consider the permissible lower limit of adsorbed magnesium to be of the order of five per cent of the total cation exchange capacity, and for potassium two per cent of the total cation exchange capacity. These are median values which are to be modified in accord with types of clay mineral and complementary ions. For example, if the dominant clay mineral is montmorillonite and the complementary ion is sodium, the clay complex must be at least 50 per cent saturated with calcium in order to meet the optimal needs of plants. At the other extreme for calcium, if the dominant clay minerals are kaolinitic and have hydrogen rather than sodium as the dominant complementary ion, 20 per cent saturation with calcium appears adequate to provide the calcium needs for growing plants.

Interrelations between potassium and calcium plus magnesium.—When plants are given potassium supplements the ratio of potassium to calcium plus magnesium is invariably increased, not only because of the higher content of potassium appearing in the plant material but also because of a reduced calcium and magnesium content. Since potassium is a commonly used fertilizer element, there are possibilities of inducing calcium and magnesium deficiencies in plants brought about by a tendency to exclude calcium and magnesium. Generally speaking, the sum of potassium, calcium, and magnesium per unit weight of a given plant is reasonably constant. Some of the work associated with these phenomena is reported. Hashimoto (3) found with soybean plants that the magnesium, calcium, and potassium in plants did not show typical antagonistic balance in an alcohol soluble fraction, but was very significant in extracts made with two per cent acetic acid. Johnson, Davis & Benne (4) in correcting magnesium deficiency symptoms in celery obtained effective control by spraying with 10 pounds of magnesium sulphate per acre at 10-day intervals. They also noted that the use of potassium fertilizers increased the incidence of magnesium deficiency symptoms. When the degree of saturation of the soil was less than 22 per cent, magnesium deficiency symptoms were enhanced. It appears that the rapidly growing celery plant makes a greater demand upon the magnesium supplies of soils than is ordinarily the case with other crops. Wander & Sites (5) demonstrated with lemon seedlings that the uptake of calcium, magnesium, and potassium was decreased when ammonium ions were supplied to the culture solutions. This suggests that ammonium as well as potassium competitively inhibits the uptake of calcium and magnesium and that the ammonium ion competes directly with potassium. An interesting observation along similar lines has been that some imported rice straw was brittle and not suitable for the making of

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straw carpets [Hashimoto (6)]. He traced this to a higher calcium and magnesium content (and lower potassium content) than was characteristic of straw from local areas. Takahashi & Yoshida (7) have also shown that high level supplies of potassium and calcium induced magnesium deficiency. Magnesium deficiency occurred with molar ratios of potassium to magnesium higher than eight. Rosselet (8) in conducting bioassays for available potassium, phosphorus, and magnesium with *Aspergillus niger* found that sufficiency levels for magnesium could not be determined in high potassium soils.

Bolhuis & Stubbs (9) showed the well-known requirement of calcium for fructification of the peanut plant to decline when potassium or magnesium was present in excess of calcium. Brown & Wadleigh (10) report that iron chlorosis induced by sodium bicarbonate is related to the $(Na+K)/(Ca+Mg)$ ratio and that the total iron content was not greatly influenced by the level of sodium bicarbonate. It is their suggestion that bicarbonate-induced chlorosis seems to depend upon the maintenance of ionic balance. Recently, considerable attention is being turned to the possible significance of the cation exchange capacity of roots as having possible implications in the plant's ability to absorb cations. Wallace & Smith (11), using different citrus root stocks and measuring the potassium and calcium contents of citrus leaves, have found that the leaf contents can be expressed by $(CEC)_1/(CEC)_2=(Ca_1/Ca_2)^2=K_2/K_1$ where CEC, Ca, and K are the cation exchange capacity and the Ca and K concentrations. Also, for magnesium $(CEC)_1/(CEC)_2=Mg_1/Mg_2$. From these observations they conclude that magnesium is subject to a different binding mechanism than calcium and further, that roots having a high charge density tend to accumulate bivalent ions in contrast to roots of low charge density differentially absorbing univalent ions. Smith & Wallace (12) report further that Ca^{45} uptake is not appreciably affected by potassium when the bean or barley plant is the experimental material.

Embleton, Jones & Kirkpatrick (13) have investigated the effects of dolomite, potash, and phosphate fertilization on the composition of Valencia orange fruit and have shown that with potassium applications, potassium content increased in the juice and peel, and calcium decreased in the peel. Citric and ascorbic acids were increased in the juice. Similarly, the same authors have shown that potassium applications increased yields along with increased potassium and reduced calcium content of citrus leaves [Embleton *et al.* (14)]. Mulder (15) has demonstrated that oat plants supplied with nitrogen from potassium nitrate or calcium nitrate on potted soils of given magnesium content did not show magnesium deficiency, whereas supplying nitrogen as either ammonium sulphate or ammonium nitrate resulted in severe to light magnesium deficiency. The deficiency could not be explained by a lowered soil pH and it was assumed that the magnesium deficiency was caused by the antagonism of ammonium ions to magnesium uptake. In the field, the results were confirmed with oats, potatoes, and corn over a period of

10 years where potassium ions from KNO_3 were similarly antagonistic to the absorption of magnesium.

De Kock (16) claims to have demonstrated that addition of potassium to chlorotic leaves caused the re-establishment of favorable phosphorus-iron ratios. It was his further observation that chlorotic plants have high phosphorus-iron ratios and low calcium-potassium ratios and that this is generally true whether the chlorosis is a simple iron deficiency or lime induced. Boswell & Parks (17), in studying the mineral composition of the corn plant in relation to soil potassium levels, used five different corn hybrids. It was their finding that the calcium and magnesium contents of leaf tissue were generally related to the potassium content and that the sums of the percentages of potassium, calcium, and magnesium contents of the plants were essentially constant at all stages of growth. Kahn & Hanson (18), also studying calcium and potassium interrelations of corn and soybean roots, have shown that calcium increases the affinity between potassium and a postulated carrier. It is their contention that there are two independent reactions and that the second one decreased the velocity of the metabolic phase of potassium uptake. Martin & Ervin (19) working with the avocado tree report that high exchangeable potassium or sodium reduced calcium absorption without, however, affecting the tree growth. It seems that a general principle is involved wherein the potassium ion competes consistently with calcium and magnesium when plants are absorbing these macronutrient cations. The phenomenon is general, having been demonstrated for a wide variety of plant species and a wide variety of cultural conditions.

Calcium in relation to cell elongation.—Calcium seems to be one of the strangest of all the macronutrients because of the great amounts needed in soils to supply plant growth. Fortunately, a great number of limestone deposits assures an abundance of this element. Even so, its functions in plants do not seem to have attracted the attention that might have been desired. A function for calcium in plant nutrition has been assumed to be the formation of calcium pectate in the cell wall, giving to the wall its characteristic rigidity. More recently it has been shown that the killing of cells with 2,4-dinitrophenol resulted in a slight increase in permeability [Ordin & Bonner (20)]. From these experiments, it was concluded that the cell membrane rather than the wall is the principal barrier to water movement. Cooil & Bonner (21) later used *Avena* coleoptiles to study growth rates as influenced by $CaCl_2$, $SrCl_2$, $MgCl_2$, and KCl. Elongation rates of the coleoptiles were inhibited greatly by the calcium and strontium salts and less so by the magnesium salt. Growth inhibition was reversed slowly by calcium-free solutions containing indoleacetic acid. However, with potassium salts added, the reversal was much more rapid. Inhibition of growth corresponded to the time required for calcium to complete occupation of exchange sites and the "outer free space" of the cells. If both potassium and calcium were present in the medium, the coleoptiles grew normally. Also, coleoptiles in the presence of

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$CaCl_2$ and sucrose increased their internal osmotic pressure greatly above sections in sucrose alone, but not when KCl was added. Thus, wall elasticity was decreased and turgor pressure was increased when immersed in $CaCl_2$ solutions. Burström (22) proposes that root elongation is a two step process consisting of plastic stretching followed by cell wall formation in which the stretching is amplified by indoleacetic acid but inhibited by coumarin. Cell wall formation is promoted by calcium. Indoleacetic acid usually promotes cell elongation at low calcium levels. Weinstein *et al.* (23) have related the growth promoting properties of the di-sodium salt of ethylenediaminetetraacetic acid (EDTA-Na) to its effectiveness in chelating calcium. Floating sections, of 2 cm. length, cut from hypocotyls of white lupine seedlings, were treated with EDTA-Na at concentrations ranging between $10^{-5}M$ and $10^{-4}M$. In 24 hr., 30 to 50 per cent increases in length and water uptake were obtained from either indoleacetic acid at $1\times10^{-4}M$ or EDTA-Na at $5\times10^{-4}M$. Other experiments using ferric EDTA-Na at $10^{-4}M$ concentration resulted in 10 per cent decreases in section length, accompanied by 15 per cent water loss. It seems reasonable to conclude, with these investigators, that the growth-promoting properties of EDTA-Na are due to its complexing with calcium in cell wall material.

Cell growth, however, cannot be entirely compatible with the idea that calcium inhibits elasticity and subsequently elongation. Indeed, quite the opposite is true for very low calcium levels. It is understood commonly that without calcium, plant roots will not extend. Ekdahl (24) has demonstrated this latter feature with roots of wheat plants. He has found that in the presence of a calcium salt, six times the elongation of root hairs is produced over that from a medium composed only of quartz distilled water. Also, root growth was reduced in the presence of oxalate, but enhanced greatly as the pH of the nutrient medium was increased. It would appear that hydrogen ions are the dominant inhibitors of root growth at these low salt concentrations and that either hydroxyl or calcium ions are effective in removing them.

Calcium salts as agricultural chemicals.—Because of the large quantities of lime which are used as an agricultural chemical, it has become important to know its fate in soils. Lysimeter studies are one of the ways of revealing gross gains and losses over long periods of time. Shaw, MacIntire & Hill (25) have completed a 20-year lysimeter experiment on the conservation of calcium and magnesium from applied burnt lime, limestone, dolomite, and dicalcium silicate as influenced by rate and frequency of liming and zone of incorporation. Such experiments are long and involve much labor. However, because of the many complexities involved in the interrelated chemistry of the product and the soil to which it is added, the lysimeter is perhaps the best instrument for getting firm ideas of the long-term process. These authors present a new concept of lysimeter soil as an interchange system wherein due account is given to the adsorption complex and the electrolytes that supply the replacing cation. Neither total outgo nor net outgo exhibited any con-

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stant relationship for losses of calcium and magnesium. Much remains to be done before enough information becomes available to enable one to predict the fate of large quantities of these types of materials when they are added to soils.

The practice of liming soils has held many puzzles for agronomists, plant physiologists, soil scientists, and others. We are all familiar with the term of "lime-induced" deficiencies. Moreover, there have been many times and many places where addition of lime has corrected deficiencies not through the provision of calcium nor adjustment of soil reaction, but rather through the impurities carried in the crude liming materials. The reviewers have had many occasions to observe spectacular field responses that were due not to the macronutrient elements presumably applied with fertilizers, but rather to zinc, molybdenum, sulfur, and magnesium. Much closer attention is being paid now to the total compositions of crude agricultural chemicals. For example, Runnels & Schleicher (26) report quantitative spectrographic determinations for 288 samples of Kansas limestones with respect to their contents of bromine, boron, chromium, copper, manganese, molybdenum, lead, nickel, silver, tin, titanium, vanadium, and zinc. Shima (27) reports analyses of limestones of the same geological era and from the same quarry and concludes that the minor elements are not related to clay, $CaCO_3$, $MgCO_3$, or phosphate components. These are some examples of complexities always associated with the subject of macronutrient elements.

Sulfur

As agriculture progresses toward greater utilization of fertile soils and moves increasingly toward lands of poorer quality, sulfur deficiencies appear more frequently. There have been many inadvertent applications of sulfur in large quantities through the use of superphosphate where sulfuric acid is used to ameliorate the rock phosphates. In keeping with recent trends to lower costs of transportation, high analysis fertilizers are replacing the crude products of former years. This may be one reason for the higher observed incidence of sulfur deficiencies. Sulfate ions are not retained well by soil colloids and are easily leached away. One is inclined to wonder how sulfur is maintained in regions such as tropical rain forests. It would seem that the biosphere must be the principal agency for holding it against the forces of leaching. It must be presumed that a continued supply over long periods of time must come from the ocean via the rains. Since the chlorine content of rain waters can be the only explanation for continued chlorine supplies in many geographical regions, there follows the logical assumption that sulfate occurring in ocean waters at one-tenth the equivalent strength of chloride must also be acquired regularly. Kurmies (28), in lysimeter experiments extending over three years, has estimated the annual loss of sulfur in Germany to be 150 kg. SO_3 per hectare. About 48 kg. per hectare are added in fertilizer of one kind or another and 170 kg. per year are added by atmospheric precipi-

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tation. He concludes that the balance of sulfur status in German soils shows a surplus of 36 kg. of sulfate per year. Egner (29) points out the great difficulty in establishing reliably the amount of nutrients received by the soil from the atmosphere, particularly as related to sulfur. Industrial smokes sometimes provide abnormally high concentrations in regions nearby the operation. Gericke & Kurmies (30) point out that determinations of SO_3 in an industrial region (Essen) showed up to 1200 kg. SO_3 per hectare yearly. They have further estimated that SO_3 originating from all the coal combustion would be about four million tons, whereas 4.9 million tons is the total amount received by soils. Mann (31) reports experiments in England involving 50 years continuous cropping, in which the sulfur received by the soil from rain and other atmospheric sources was ample to maintain the sulfur content of the soil even though not fertilized in any way. Sulfur is found in combination with humus and it is his suggestion that it would not tend to be carried downward to the subsoil. Kreger (32) offers another possible mechanism for retention of sulfur, in demonstrations of x-ray diffraction patterns of $BaSO_4$ found in fungi and algae—*Saccharomyces cerevisiae, Mucor rouxianus,* and *Spirogyra.* Where soils are waterlogged, as in rice culture, reduction of sulfur compounds to sulfides takes place very rapidly and the very insoluble iron sulfide compounds are formed. Desai, Seshagiri Rao & Hirekerur (33) point out that sulfide toxicity may result in poorly drained rice fields when sulfates are used in fertilizers. Olsen (34) working with cotton plants has demonstrated that 10 p.p.m. of sulfate in culture solutions is required for the plants to reach maximum growth. Also, sulfur derived from the atmosphere was an important fraction of the total needed by plants, with the larger plants acquiring the greater amount of sulfur from this source. Where the sulfur supply in cultures was restricted, over 50 per cent of the total acquisition was from the atmosphere. However, atmospheric sulfur could not (under the conditions of his experiments) supply total plant needs.

The pattern which is emerging is that sulfur will have to be supplied in ever increasing quantities from mines. For example, Martin (35) reports widespread sulfur deficiencies in the State of California with 34 of its 58 counties being involved. This is a large geographic region where a few years ago sulfur deficiencies were scarcely noted.

Phosphorus

It is fitting that this section should be introduced with a recent report from the Rothamsted Experimental Station on data from experiments started by Lawes and Gilbert 75 years ago. Warren (36) reports that phosphorus residues from superphosphate in manure applications made in the middle of the nineteenth century have increased the yield of barley by 7 to 10 cwt. per acre, 50 years after the last application. The rate of recovery of the phosphate applied so long ago has been 0.5 per cent of the total phosphorus applied earlier from a variety of sources, including superphosphate and manure. It

is the reviewers' contention that if there were to be candidates for "individuals having made the greatest impacts upon mankind" Lawes and Gilbert would be outstanding candidates. Their demonstration and experiments showing that mineral phosphorus could be attacked by sulfuric acid in a way to restore rock phosphate to the plant world, opened the way for expanding agriculture with the wedding of industrial activities to the production of foods so that since that day science, industry, and agriculture have all advanced together. It is unique in science that experiments outlined and begun over 100 years ago are still active and still yielding data of significance. It is to be hoped that they will remain for centuries to come, continuing to point out that the agricultural activity of the day is dependent in some part on all that has gone before. Agriculture is a long-term process and there will always be need for laborious, painstaking, experimental work in the field of the kind that Lawes and Gilbert have exemplified. As an example of recent work, Robertson & Hutton (37) report upon evaluation of 10 different sources of phosphate fertilizers as reflected in the growth of field crops. Their experiments were conducted for a period of six years with corn, oats, peanuts, and soybeans. The experiments were designed to show differences between limed and unlimed soils in their abilities to supply phosphorus from the 10 different fertilizer materials. Recovery studies with the radioactively tagged phosphorus, P^{32}, has led these authors to suggest that some of the responses obtained in the way of higher yields might have come from contaminating micronutrient elements.

In relation to macronutrients required by land plants, it is of interest to comment on the role of phosphorus in sea waters. It is a popular assumption that the oceans in their vast extent could become an inexhaustible medium for growth of food plants. The great barrier to ideas of this sort is the extremely low phosphorus content of ocean water. It is the low phosphorus status that limits plant production now. Should we wish to consider increasing the yields, one of the first things to think about would be supplementation with phosphorus from other sources. Steele (38) has sampled the oxygen content of ocean waters in the North Sea and compared the phosphate concentrations as a measure of plant production. It is his finding that a direct conversion factor of 1 μgm. atom of phosphorus is equivalent to 3.87 ml. of oxygen. Consequently, the limiting factor is phosphorus so that the amount of photosynthetic activity possible in these ocean waters is directly connected with the amount of phosphorus available. Steele (39) has also studied the photosynthetic activity directly with the radioactive carbon isotope, C^{14}, as a measure of plant production in the sea and has compared these data with the inorganic phosphorus data for the same North Sea region. There was quite good agreement between the two methods. The amount of carbon fixed per square meter during the summer months was in the order of 0.2 gm. of carbon. If one compares these data with accepted production levels of land plants under managed agriculture, an equivalent area of the

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sea is only about one per cent as efficient as the land in the production of organic life. When one realizes that the limiting factor here is phosphorus, it becomes evident that "ocean farming," when and if developed, must devolve upon gathering in the phosphorus of the sea water to the crop intended for harvest. Abbott (40) in studying the growth of algae in Lake Houston, Texas, where the phosphate concentration is less than 1 μgm. per liter, suggests that algal phosphorus is obtained from colloidal clay coming from run-off waters. It is to be observed that all bodies of water are extremely low in phosphorus content and consequently the amount of plant life that they can support is restricted. There are exceptional cases, of course, where sewage is added to waters or where the water is in close contact with land, as in shallow marshes.

Interrelations between phosphorus and other macronutrients.—Soils are the usual medium for plant growth and must always supply nitrogen along with phosphorus. The soil chemistry of these two macronutrients is closely related in a way that influences the availability of phosphorus to growing plants. The further fact that the dominant macronutrient cation in soils must be calcium, means that the little-soluble compounds of calcium-phosphate are always to be considered as being under the influence of the calcium in soils, with solubilities directly affected by degrees of acidity. Lorenz & Johnson (41) have demonstrated very clearly that the source of nitrogen, whether from ammonium or nitrate salts, can make the difference between highly successful crop production or crop failure of potato plants when grown on light, slightly alkaline soils. The reason is that the physiologically acid ammonium salt releases enough native soil phosphorus to supply the full plant requirement compatible with the growth potential made possible through supplying the extra nitrogen. Nitrate fertilizers are physiologically alkaline so that when salts such as calcium nitrate or sodium nitrate are used, the solubilities of the native soil phosphates are reduced to a level below that needed by the growing plant. Olson & Dreier (42) using wheat and oats as indicator crops to test the action of nitrogen in promoting utilization of phosphorus, report ammonium salts to be superior to nitrate salts, especially during early stages of growth. Another interesting type of biological acidulation under field conditions has to do with the addition of sulfur along with rock phosphate as reported by Neller (43). He has shown that plantings of oats or clover removed 77 per cent of the phosphorus from the rock phosphate in sulphured soil. It is to be presumed that the biological activity oxidizing sulfur to sulfate ion was highly effective in rendering available the exceedingly insoluble rock phosphate.

Absorption of phosphorus by plants from soils and solution cultures.—Soil phosphorus levels are always very low. If the soil solution contains as much as 1 p.p.m. phosphate, the value is considered high. There are many instances where the soluble phosphate is very low, e.g., in the order of 0.001 p.p.m. If

plants extract phosphorus from the soil solution only, it is quite clear that the phosphate in the soil solution has to be renewed very frequently and continuously from the solid phase. Simple calculations show that for many situations in the field it would be necessary for the soil solution to be completely renewed as many as 100 to 500 times during a single growing season. Consequently, the volume of the soil explored by roots becomes highly important as does the rate of renewal of phosphate from the solid phase.

Fried & co-workers (44) have proposed that the kinetics of phosphate renewal may be of much less importance than limits imposed by the rates at which the plants absorb the phosphate. They have demonstrated that 13 to 15 pounds of phosphorus per acre can be released each hour. This exceeds the rate of absorption by the plant by a factor of several hundred. Rates of renewal showed high temperature coefficients as are characteristic of chemical reactions. From this they have concluded that soil systems are usually at equilibrium with the solid phase. Jacobson, Hannapel & Moore (45), in experiments with excised roots and intact plants, have concluded that nonmetabolic uptake of HPO_4^{--} is equivalent to the apparent "free space." Because of lower values for nonmetabolic uptake for this phosphate ion by intact plants over that of excised roots, they have proposed that a barrier exists between the stellar region and the cortex. It would be this barrier in roots which prevents movement of ions by mass flow from the external solution into the conducting elements of the roots.

With reference to the importance of having knowledge of the amount of soil volume explored by roots and the acquisition of phosphorus therefrom, there are new studies by Lipps & Fox (46). They report that long-time alfalfa cropping on deep soils has resulted in measurable reduction of total and available phosphorus to a depth of 11 feet. If soil solutions are the important source of phosphorus for growing plants, the condition of soil moisture is another complicating factor within the total system. Dean & Gledhill (47) have investigated phosphate absorption by excised roots placed in contact with dry soil and found that phosphorus can pass from soil to roots at the same time that water is passing from roots to soil. In order to accomplish these experiments, they used radioactive phosphorus and preconditioned the roots to high or low moisture stress before bringing them into contact with the dry soil. Movement of phosphorus into roots preconditioned at high moisture stress was at a reduced rate compared with that into roots preconditioned at low moisture stress. Harris & Van Bavel (48) have called attention to carbon dioxide as a further contributor to the plant-soil interrelationships involving phosphorus. They have demonstrated with tobacco plants that in general, nutrient uptake remained relatively constant over a wide range with respect to oxygen content of the soil atmosphere, until such concentrations fell below 10 per cent. If carbon dioxide increased to five per cent of the soil atmosphere, however, the uptake of phosphate was

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increased. In connection with conditions within plants themselves which would explain something of their power to assimilate phosphorus, we have the interesting suggestions of Berezovskiĭ & Kurochkina (49). They have shown that spraying sunflower plants with 2,4-D resulted in lower uptake of phosphorus from the soils, with the phosphorus tending to collect in the roots at the site of entry. Also, if 2,4-D was introduced through the roots, the inorganic phosphorus did not tend to rise in the plant.

Flaig & Kaul (50) have studied the uptake of phosphate from the large organic phosphorus compound, nucleic acid. Young cereal seedlings took up phosphoric acid, but the reaction was determined by the dephosphorylation of nucleotide. Nucleosides remained in the nutrient solution. When phosphate has once entered the plant, however, organic compounds may be formed and translocated [Tolbert & Wiebe (51)]. These investigators, using P^{32} and S^{35}, examined chromatographically the exudates from the stumps of decapitated barley, tomato, sunflower, bean, and willow plants. Besides inorganic phosphorus, there were two unknown phosphorus-containing compounds, with as much as 20 per cent of the total P^{32} being in the unknowns. S^{35} is mentioned at this point because it was found only as sulphate. The unknown phosphorus compounds are considered as normal constituents of xylem sap. Rothburr & Scott (52) suggest that metabolic activity related to the absorption of phosphate is connected also with silicate. Using young wheat plants in culture solutions, they have shown that added silicate enhances absorption of phosphate and, conversely, that added phosphate depresses slightly the uptake of silicate. As with phosphate, once absorbed, silicate (as revealed with Si^{31}) was found to be distributed generally throughout the plant within half an hour. Michael & Marschner (53) have studied in greater detail the traffic from solution to plant and plant to solution. Using P^{32} they grew wheat plants labeled with the radioactive phosphorus isotope. Later they measured its passage outward when the plants (after rinsing) were transferred to cultures containing untagged phosphorus. They found the maximum exchange rate at from 20 to 25 minutes, decreasing interchange continuing for two to three days. Phosphorus-rich plants gave off larger absolute amounts of radiophosphorus, but percentagewise, the phosphorus-poor plants effected the greater exchange. The diffusion outward was small, amounting to about 0.2 per cent of the total phosphorus contained in the plants; 70 per cent of the released radiophosphorus was inorganic phosphorus and 10 per cent phosphatide. Experiments with metabolic inhibitors such as KCN, 2,4-D, or CO_2, increased phosphorus excretion almost linearly. Pleshkov (54) may have brought forth suggestions that would serve to explain in part the persistently recurring impressions that under some circumstances phosphates can be toxic to plants. He has grown corn plants on phosphate-free media which results in excessive amounts of amides and other nonprotein nitrogen substances being synthesized. Introduction then of phosphate is

followed by enhanced rapid synthesis of protein, amide, and nonprotein substances, and increased uptake of ammonia. Ammonium ion then left over is claimed to have accumulated in the tissues in concentrations sufficient to produce toxicity. Similarly, plants grown with nitrate as the nitrogen source are presumed to reduce nitrates to ammonia in sufficient quantity to produce toxic effects. Actually, this latter supposition appears to the reviewers as needing much more evidence, if it is to be accepted as a mechanism to explain "phosphorus toxicity."

Among the highly important questions which face farmers, agronomists, and research workers alike, is whether or not there are real and fundamental differences among plant species, or varieties, in their abilities to absorb phosphate from soils. From casual evidence or ordinary observations, this would seem to be so to a high degree. For example, on acutely deficient phosphorus soils one can fully expect to plant a crop of buckwheat and have something to harvest. Should a tomato plant be grown upon the same soil, it may never attain a height of more than a few inches with the possibilities of no blossoms or setting of fruit being obtained. Nevertheless, if one designs an experiment for the purpose of delineating clearly that the buckwheat plant is capable of extracting more phosphorus from the soil than the tomato plant, the results would be negative or inconclusive if they were to follow the pattern of the average experiment directed toward ascertaining whether or not some kinds of plants really do have a greater ability to extract phosphorus from low-phosphorus supplying soils than other plants. Lawton & Davis (55) may have shown a species difference between corn and Sudan grass in their observation that corn absorbed many times the amount of phosphorus as compared to Sudan grass. Even so, the fact that the percentage of fertilizer-derived phosphorus in the two crops was the same for all lime-superphosphate applications implies that the observed differences might be due only to rates of absorption rather than a greater ability of the corn plant to mobilize insoluble phosphorus compounds in soils.

Pratt, Jones & Chapman (56) have reported upon the results of 28 years of differential fertilization in an irrigated soil planted to citrus. Both the total and soluble phosphorus showed significant changes in the soil. The total phosphorus had decreased at all depths to 36 in. From 36 to 48 in. no significant changes were observed. The soil levels which were affected were the same, whether or not phosphorus had been added. With phosphorus fertilization, increases in soil phosphate were observed and 60 per cent of the additions could be accounted for in the first 6 in. of depth. The first foot of soil accounted for 80 per cent of the total amounts of phosphorus added. Auxiliary fertilizer treatments that acidified the soil produced increases in the $NaHCO_3$ soluble phosphorus. Shapiro (57) has shown that flooding of rice resulted in increased yields due to added availability of phosphorus. This observation is in keeping with straightforward chemistry of sparingly soluble

salts, in that the solubility product of calcium phosphates should require larger amounts of phosphorus coming into solution upon the dilution of calcium ions. The clue is that flooding increased the availability of soil phosphorus but remained without effect on soil-nitrogen availability.

Hunter & Thornton (58) have made one of the most novel suggestions of a source of phosphorus fertilizer as yet conceived. It is their suggestion that phosphine (PH_3), though extremely toxic to animal life, including humans, could be handled as a fertilizer with no more difficulty than we have been accustomed to assume in connection with the more potent phosphorus-containing insecticides. They have supported their argument with experimental demonstrations that phosphine can be added to soils without loss; that higher plants, such as radishes and wheat, are not affected deleteriously except at rates well beyond the amounts of phosphorus normally required in fertilization practices; that the availability to plants of the phosphorus contained in phosphine is almost double that of superphosphate; and that the weight of the phosphorus-containing material that would have to be transported as a fertilizer would be only one-tenth that of one of our contemporary "high analysis" fertilizers. Curiously enough, using our archaic method of expressing amounts of phosphorus in fertilizers on the basis of P_2O_5, phosphine contains 209 per cent P_2O_5. Other arguments, based on chemistry, are good ones in that the phosphorus in the form of phosphine can be handled and applied in a way analogous to ammonia (NH_3). We may look forward to further experiments with this interesting phosphatic material and to the effects that it might have on the microbial population of soils as well as the curious ability of plants being able to tolerate this chemical compound which has such an extreme toxicity for animals. Hopkins (59) has reviewed the latest developments pertaining to the use of phosphine as a phosphatic fertilizer as of 1956. The reviewers have not been able to discover further reference to this subject.

In concluding these remarks on the subject of phosphorus as a macronutrient, it is to be recalled that the importance of determining the phosphorus supplying power of soils by chemical means in the laboratory cannot be underestimated. McDonnell (60) calls attention to an obvious, but hitherto disregarded, source of error in making chemical tests of the phosphorus status of soils. He points out that in county Galway, Ireland, a deficiency of phosphorus exists in calcareous grassland soils in spite of high analytical values for readily soluble phosphorus. He has traced these high values to shells of *Helicella itala* and *Helicella aspera* which were present in the soil. The amounts of phosphorus in soils are so exceedingly low in the soluble forms that one can readily see how a small bit of snail shell could confound the analytical results obtained in the chemical laboratory, which are supposed to be interpretable in terms of acres of land.

Potassium

For many years potassium has been the mysterious macronutrient. For all of the other macronutrients, incorporation into the structures of proteins, carbohydrates, prophyrins, and analogous components of living matter, make their uses understandable. With potassium, there are no such compounds known. Even if there were, there would always remain the question as to why a similar monovalent cation, such as sodium, should not be able to accomplish the same functions as potassium. We are familiar with the term, "partial substitution of sodium for potassium functions" especially as it applies to the beet plant. However, in spite of a great many attempts to examine the sodium need of plants, even calling upon the greatest skills engendered by work with the micronutrient elements, there have been so far only two findings that sodium is really a nutrient element. Also, in both of these cases, it has to be classed as a micronutrient rather than a macronutrient. Brownell & Wood (61) have reported that *Atriplex vesicaria*, a halophyte, does indeed require a small amount of sodium for its growth and development. Allen & Arnon (62) also have shown sodium to be a micronutrient required for the growth and development of *Anabaena cylindrica*, an alga. No one has ever been able to demonstrate that any kind of plant can be grown without potassium, in spite of attempts to substitute sodium or rubidium. For many years, the idea has been expressed that potassium is needed in order to form carbohydrates. This has never been a satisfactory kind of statement because one could just as well say that without potassium plants do not grow, or that potassium is an essential element. There are a few reports now appearing which tend to give credence to the idea that carbohydrates are involved with potassium nutrition. These are observations that at low potassium levels respiration is increased. Mulder (63) reports that potatoes grown under deficient nutritional conditions show higher rates of respiration than potatoes with high potassium. Moreover, these effects being observable with the potato tuber, it is possible to conduct better controlled experients than is normally possible for studies with growing plants in the field or in culture solutions. Further to the point, we have so often heard from people handling agricultural products in transit to points of distribution, that there are differences in quality as evidenced by the ability of some products to hold up better than others in their postharvest stage. Perhaps a way has been shown that will permit studies eventually leading to the real understanding of the functions of potassium in plant nutrition. Fujiwara & Iida (64) also point out that decreased potassium supplies resulted in increased respiration of plants and that such respiration was not affected by sodium. They also state that plants having had adequate supplies of potassium showed greater resistance to unfavorable environments, as well as having a larger amount of carbohydrates. Hofmann (65) reports that

activity of phosphoesterases was increased by potassium and presents this evidence as being in agreement with the idea that potassium is linked somehow in the formation of carbohydrates. The implication is that the enzymes which break down carbohydrates are inhibited by higher amounts of potassium. His studies were made from enzyme preparations prepared from the leaves of potatoes, spinach, and wheat, as well as extracts of potato tubers, which had been grown with various nutrient supplies.

Plant-soil interrelations involving potassium are no less in the category of incomplete characterization. Ideas from general chemistry would stress potassium as an element forming, with few exceptions, highly soluble compounds. In soil chemistry, however, there are a number of expressions which have crept into our technical language because of the desirability of calling specific attention to particular types of reactions between potassium ions and soil systems. For example, in addition to the intracrystalline potassium of insoluble aluminosilicates such as feldspars, micas, and biotites, there are these terms: "soluble potassium," "exchangeable potassium," "nonexchangeable potassium," "potassium rendered nonexchangeable upon drying," and "nonexchangeable potassium released upon drying." Over the years there have been repeated observations that plants are capable of extracting potassium from soils that one cannot extract quickly or easily in the chemical laboratory. Only by resorting to very drastic treatment as compared to anything that a plant could do are we able to secure from soils amounts of potassium that are equivalent to those extractable by plants over long cropping periods. Mortland, Lawton & Uehara (66) have made an outstanding contribution to the understanding of plant-soil interrelations involving potassium nutrition with their observation that the plant can be responsible for altering the lattice structure of a soil mineral even within the short time of a greenhouse experiment. They have shown that biotite supplied as the only source of potassium to crops was altered to vermiculite. Their evidence of this rather startling pronouncement seems unequivocal. The appearance of a 14 A unit diffraction line characteristic of vermiculite in a growth medium formerly consisting only of biotite having a spacing of 10 A units is rather clear evidence that the plant is able to create new mineral species within very short periods of time. Matthews & Smith (67) have devised an apparatus for continuous circulation of water through a soil sample held in series with a cation exchange resin. The objective of their experiment was to produce a system capable of maintaining the solution in contact with soils at the lowest possible level of potassium. It is presumed that in this way they had hoped to simulate one of the characteristics of plants which is an ability to extract and hold potassium against a concentration gradient, having a fractional parts per million concentration external to the plant root. They found that the amount of exchangeable as well as nonexchangeable potassium released from the soil and adsorbed by the resin correlated highly with the amount of

potassium taken up by alfalfa plants when grown continuously with a limited amount of soil in greenhouse pot experiments. Rates of release of nonexchangeable potassium showed little correlation with the original levels of exchangeable potassium for individual soils. However, it was shown that the rate of release of potassium was an individual characteristic of given soils whose suitability for supplying potassium was determined best by the plant, and secondly, by a chemical process such as the one devised, which was capable of removing potassium continuously from material supplying potassium at very low levels, namely, in the fractional parts per million class. Garman (68) has examined 17 Ohio soils, 25 New York surface soils, and 21 New York subsoils for their potassium release characteristics when subjected to continuous cropping. The potassium which was extracted by plants was measured and compared with chemical methods applied to the same soils. Garman found that a continuous leaching method with 0.01 *N* HCl gave the highest correlation of potassium extractable by chemical methods with that by continuous cropping. Pope & Cheney (69) have also done similar experiments and have used chemical extractants of more reactivity—vastly beyond anything that a plant could produce. For 20 different soils representing seven different soil series, they found that boiling in 1.0 *N* HNO_3 gave the best correlation with extractable potassium between the activities of the plant over long-term cropping and the procedures of the laboratory. These references pertaining to the extraction of soil potassium by chemical means are but a small fraction of the total effort being directed toward devising reliable chemical methods for predicting crop responses to the potassium contained in soils. These references have been selected because of their boldness of approach coupled with a sufficient number of soils of different natural characteristics to make the results of the studies acceptable for purposes of generalization.

Foliar Absorption of Macronutrients and Mobility of Macronutrient Elements in Plants

Leaves of plants have large surface areas. The amount of water which they transpire approaches that of the water absorbed by roots. Moreover, the carbon dioxide assimilated by leaves contributes the greatest fraction of the dry weight of the plant. It seems reasonable, therefore, to view the plant leaf as an organ having possibilities for absorption of nutrient elements besides carbon. With the development of spray machinery, it has been possible to treat large acreages of growing plants within a matter of hours. Consequently, there remains persistent interest in possibilities for using spray methods to fertilize plants [see (4)]. Wittwer (70) has reviewed the subject of foliar absorption up to 1956 in a paper containing 92 references. He points out that radioisotopes have been more useful in studies of absorption and transport of nutrients than any other application they have had in problems

relating to agriculture. With nitrogen, however, there are no suitable radioisotopes for long-term experiments, but the rare, stable isotope, N^{15}, has proved to be an adequate substitute except for the technical difficulties of doing the necessary analytical measurements. Burr & Takahashi (71) have studied the absorption and distribution of nitrogen in sugar cane following its application as urea to the leaves. A single spray treatment introduced an appreciable amount into the plant. They have reported nitrogen in the sugar cane as remaining in a state of flux, with old nitrogen being exchanged for new in every part of the plant. Temperature, age, and nitrogen supply were all considered as influencing exchange rates. Their paper illustrates nicely the power of the tracer technique with N^{15} where nitrogen is to be followed over long periods of time.

The subject of absorption, transport, and mobility of nutrient elements within plants, as revealed by radioisotopes, always elicits the name of Biddulph, who has made extensive contributions to this field since the first availability of synthetically produced isotopes. Biddulph, Cory & Biddulph (72) have shown the following things about the mobility of sulfur in the red kidney bean: a portion of the total sulfur remains mobile and moves freely from one organ to another; a portion of seed sulfur, originally translocated to roots is released for upward translocation only when additional sulfur enters the plant from the root environment; and the rate of downward movement of sulfur in the phloem of the stem is in excess of 40 cm. per hr. Koontz & Biddulph (73) on the subject of absorption and translocation of foliar applied phosphorus are able to conclude that for absorption purposes: spray applications are superior to vein injection or droplet methods; more phosphorus was translocated from older leaves than from younger leaves; very young leaves did not export phosphorus; leaves contributed phosphorus to the root in proportion to their proximity to the root; leaves exporting phosphorus contributed approximately equal amounts to the stem apex; phosphorus translocated from a given compound appears related directly to drying times of solutions on the leaf; absorption of leaf-applied phosphorus amounts to 60 per cent of the application after about 30 hr.; 34.5 per cent of the applied phosphorus was translocated out of treated leaves within 96 hr.; and surface-active agents were ineffective in increasing translocation. Biddulph & Cory (74), turning their attention to water, phosphorus, and carbon and using as labeling agents tritium (THO), $H_2P^{32}O_4^-$, and $C^{14}O_2$, respectively, applied simultaneously to the surface of a bean leaf, showed that: the amount of isotope moving per unit applied was greatest for C^{14} and least for THO; C^{14} moved largely as sucrose; P^{32} moved largely as phosphate or fructose-1,6-diphosphate not containing the C^{14} label; velocities for P^{32} and THO were 87 cm. per hr.; C^{14} moved at the rate of 107 cm. per hr.; there was an approximately linear relationship between the logarithms of P^{32} and C^{14} concentrations and their distances moved from the point of entry into

the stem; there is evidence that some features within sieve tubes may restrict freedom of solute molecules to accompany the solvent; losses from sieve tubes to xylem amounted to approximately 24 per cent for P^{32} and C^{14} and 31 per cent for THO; and there must be a marked permeability of the lateral sieve tube membranes. Biddulph and co-workers (75) on "Circulation patterns for phosphorus, sulfur, and calcium in the bean plant" have concluded that: the radioactive isotope of phosphorus displayed a sustained circulation throughout the plant during the whole of a 96 hr. experimental period; S^{35} was originally mobile, but circulation was curtailed rapidly by metabolic capture in young leaves; and Ca^{45} did not recirculate following its initial delivery via the transpirational stream. Again, Biddulph, Biddulph & Cory (76) on "Visual indications of upward movement of foliar-applied P^{32} and C^{14} in the phloem of the bean stem" conclude that: movement of P^{32} occurred in the phloem tissue principally between leaves of the same orthostichy; and C^{14} supplied as carbon dioxide moved both upward and downward in phloem tissue from which there was lateral movement into the xylem.

Bukovac & Wittwer (77) have studied the absorption, transport, and mobility of five of the macronutrients: potassium, phosphorus, sulfur, calcium, and magnesium as well as all of the micronutrients except boron, applied as radioactive isotopes to leaves of the bean plant. Their criterion of transport was based on the percentage of foliar-applied radioactive isotopes recovered in nontreated plant parts. Radioautography was used to observe the gross distribution. They have concluded that potassium was the most readily absorbed and the most highly mobile of the macronutrient elements tested. Calcium and magnesium were not mobile, whereas phosphorus and sulfur were intermediate, but with phosphorus being transported more readily than sulfur. Thorne (78) has shown that ammonium nitrate applied to leaves of sugar beet plants resulted in even further increase of nitrogen absorbed by roots. In contrast, uptake of phosphorus by roots of swedes was decreased by applying sodium phosphate solution to the leaves. As with the nitrogen, potassium applied to leaves resulted in increased potassium absorption by roots, but only when the plants were cultured with a low supply in the nutrient solution. Thorne (79) has demonstrated that P^{32} was detectable in the roots of swedes or beans within three hours after application to leaves. Phosphorus was absorbed more readily from the lower surfaces of leaves than from upper surfaces. Increasing the relative humidity of the air increased P^{32} uptake; shading usually depressed it. Studies of mineral solute translocation in trees are much more difficult to perform than with smaller plants. However, progress has been made in this area. Swanson (80) has reviewed some of the work and conclusions from special studies with trees. He points out that larger trees, with distances of several hundreds of feet, magnify considerably the problems of tracing nutrients. In opposition with the sorp-

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tion of nutrients by foliar application, it has been known for many years that rain and dew leach materials away from leaves. Tukey & Amling (81) have demonstrated this leachability with 11 different radioisotopes and point out that the relative ease of removal of macronutrients from leaves is in the order of calcium, magnesium, sulfur, potassium, and phosphorus.

Nitrogen

Nitrogen is the one macronutrient present everywhere in the atmosphere. The only limitation upon supply is the rate at which microorganisms and atmospheric electricity can convert it to forms which can be assimilated by plants. Aside from the mention of nitrogen in the above text, incidental with discussion of the other macronutrients, the prime interest in this research area lies in microbiology, in assoications with the micronutrient elements, especially molybdenum, and in elucidations of biochemistry. McElroy & Glass (82) have compiled a group of significant papers on inorganic nitrogen metabolism available in a book of 728 pages.

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NITROGEN NUTRITION[1]

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Certain aspects of the nitrogen nutrition of plants have been covered in earlier volumes of the *Annual Review of Plant Physiology* (61, 74, 176, 178, 200, 205, 207), and the symposium on *Inorganic Nitrogen Metabolism* edited by McElroy & Glass (119) constitutes an excellent review of the subject to 1955. The present discussion covers only recent literature through September 1958. The subjects of nitrogen fixation (to be discussed in the 1960 volume) and plant proteins (covered in the present volume) are excluded, and other aspects of nitrogen nutrition and metabolism which have been reviewed recently have received little attention here.

Nitrogen in the Soil

A wide variety of complex nitrogenous compounds are added to the soil as residues from plants and animals. These materials are degraded, chiefly through hydrolysis and oxidation by microorganisms, to simpler compounds which are suitable for the nutrition of plants.

Nitrogenous organic compounds.—Soil constituents have been investigated by Cornfield (46) who compared the hydrolysis products released by fertilized and unfertilized soils, and found surprisingly little difference in composition in loam soils which were fertilized or unfertilized for a period of eight years. However, sandy loam, sand, and chalky sand soils were distinctly lower in nitrogenous constituents when they were left unfertilized during this period.

The availability of the nitrogen in the soil depends upon the concentration of nitrogen in the organic residue, for decomposition of a nitrogen-poor residue will support a large microbial population and will release little nitrogen for the growth of higher plants. Williams & Ririe (204) reported that barley residues decreased the production of unfertilized sugar beets by rendering nitrogen unavailable. Parker *et al.* (141) have found that the uptake of nitrogen by Sudan grass in greenhouse experiments was greater when cornstalk residues were added to the soil surface than when they were incorporated into the soil. However, in the field, the surface residue decreased uptake of nitrogen unless nitrogenous fertilizer was added, and yields of corn were depressed. With time, the differences in availability of nitrogen level off as organic matter decomposes; Laws (110) found no appreciable difference in nitrogen levels after 48 months in nitrogen-treated soils which had received red clover or wheat straw, although the initial organic matter to nitrogen ratios were 21.0 and 34.4 respectively.

[1] The following abbreviations will be used: FAD, flavin-adeninedinucleotide; FMN, flavin-mononucleotide; DPNH, diphosphopyridine nucleotide (reduced form); TPNH, triphosphopyridine nucleotide (reduced form).

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Haas *et al.* (84) conducted an extensive study of the nitrogen balance in great plains soils cropped from 30 to 43 yr. The average of all locations indicated a 39 per cent loss in nitrogen over a period of 36 yr. Losses from continuous cropping to small grains were less than under row crops or alternate small grains and fallow. Under plain conditions, crop production normally was limited by moisture rather than nitrogen.

The hydrolysis of protein residues in soil yields a variety of amino acids, and although these may be ammonified and nitrified before assimilation, it also is possible for the plants to use them directly. Many microorganisms can assimilate amino acids readily, and will compete with the higher plants for amino acids in the soil. Certain soil microorganisms also will excrete amino acids when growing on inorganic nitrogen [Payne *et al.* (142)].

Biswas & Das (13) observed cystine as the only free amino acid in the soil of a fallow plot, but in a plot growing *Trifolium alexandrinum* they also found aspartic acid, alanine, glutamic acid, leucines, phenylalanine, arginine, and threonine. Nilsson (133) observed little difference in the amino acid levels in soils under crops of oats, barley, and peas. Sowden (170) examined five soils which contained from 14 to 41.8 per cent of their nitrogen as amino acids and recorded the amino acid distribution in hydrolysates of the soils. The percentage of amino acids varied with the soil horizon, decreasing with depth, an observation also reported by Stevenson (175). Turchin (187) has determined the amino acid composition of sulfuric acid hydrolysates of soil by quantitative paper chromatography and has found little difference among soil types. Kononova & Aleksandrova (106) identified 16 amino acids in the hydrolysates of soil humic acid and concluded that proteins constitute a substantial fraction of soil humus. Bremner (21) recovered humic acid fractions from nine soils by extraction with 0.5 *M* NaOH or with 0.1 *M* sodium pyrophosphate. The two types of preparations from the same soils were quite different in total N and in their nitrogen distribution after acid hydrolysis, but amino acids constituted the bulk of the dissolved nitrogen, and proteins accounted for much of the undissolved residue.

Champigny (37) fed cuttings of *Bryophyllum daigremontianum* with C^{14}-labeled glutamic acid and found a part of the glutamic acid was incorporated unchanged. A part of the C^{14} from 1-labeled and more extensively from 3,4-labeled glutamic acid was incorporated into a variety of amino acids and fatty acids, apparently by deamination of the glutamic acid and metabolism of the products formed via the tricarboxylic acid cycle. Ratner *et al.* (150) grew corn and sunflowers under aseptic conditions and demonstrated that they could assimilate glycine, aspartic acid, glutamic acid, and arginine as the sole sources of nitrogen, although none of these was as effective as inorganic nitrogenous compounds. There was a very slow utilization of lysine, alanine, tyrosine, and guanine; phenylalanine and tyrosine alone were somewhat toxic. The ammonia level in the plant juice was unusually high when the readily assimilable amino acids were fed.

Recent work on the assimilation of amino acids by intact plants has been

limited, but their use in tissue cultures has attracted more attention. El-Shishiny & Nosseir (56) have observed a much faster assimilation of L- than of D-glutamate and of L- than of D-aspartate by carrot root disks. D-Glutamate and D-aspartate accumulated and depressed the uptake and use of ammonia nitrogen. Birt & Hird (12) also studied the uptake of amino acids by carrot tissue; the dependence of the uptake upon respiration was indicated by the fact that it was inhibited by 2,4-dinitrophenol and by cyanide. L-Histidine was taken up faster than D-histidine, and L-histidine and L-arginine were mutually inhibitory as measured by their uptake. Steward *et al.* (177) found that hydroxyproline strongly inhibited the growth of carrot phloem explants. Isolated oat embryos grew well on ammonia, nitrate, casein hydrolysate or a mixture of 18 amino acids as a source of nitrogen [Harris (85)]. Twelve amino acids when supplied singly inhibited the growth of roots; in combination the inhibitory action was partially suppressed. French & Gibson (69) found that glutamic acid stimulated the growth of the roots of *Datura tatula* on White's medium. Nitsch & Nitsch (134) observed that sections of Jerusalem artichoke tissue grew as well on α-alanine, γ-aminobutyric acid, glutamic acid, aspartic acid, glutamine or urea as on an equal concentration of nitrate nitrogen. Acid hydrolyzed casein also supported good growth, but glycine, asparagine, allantoin, and arginine were not as effective as sole sources of nitrogen. The D-isomers were not used and in some instances were inhibitory.

Bremner & Shaw (22) observed that formaldehyde-treated protein waste products decomposed more slowly in soil than the untreated proteins. Lignins treated with nitric or nitrous acid decomposed more rapidly than lignin samples treated with ammonia, whereas cellulose nitrate was very resistant to decomposition. Boquel (19) has followed ammonification in a variety of soils, and Stojanovic & Broadbent (179) observed that ammonification was about twice as fast at 10° as at 5°C.

Peas carry an enzyme which Little (113) has found rapidly oxidizes 2-nitropropane to yield nitrite and acetone. The enzyme is a peroxidase, and in common with other peroxidases its action is stimulated by manganese and certain phenols. Cain (32) has studied species of the nocardia and pseudomonas groups which can oxidize nitrobenzoic acid after adaptation to the substrate.

Fertilizers.—An abundance of tests to establish optimal concentration, placement, mode and time of application, and type of fertilizer are reported in the current literature. Only a few of these studies can be mentioned, although their practical importance cannot be denied, for the consumption of nitrogenous fertilizers has increased at a spectacular rate in recent years.

Olson & Dreier (137) found that the uptake of fertilizer phosphorus by wheat or oats was stimulated up to twofold by nitrogen fertilization under a variety of conditions. The effect of nitrogenous fertilizers was attributed in part to their stimulation of root activity. Bullen & Lessells (25) have reviewed their extensive studies of the effect of nitrogen on the yield of cereals,

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and have recommended fertilizer levels for wheat, barley and oats. Widdowson & Cooke (203) found drilling was more beneficial than broadcast application of ammonium sulfate for barley and wheat. With a 50 pounds N per acre application, they recovered a third of the nitrogen in barley grain. Burleson *et al.* (26) found good increases in protein of sorghum grain and forage when 60 or 120 pounds of nitrogen per acre was supplied. Their apparent recovery of added nitrogen was 83 to 90 per cent.

Pasture lands often are neglected, but the yield, protein content, and seed production of forage grasses all were improved in Carter's (35) experiments with nitrogen fertilization. Autumn application appeared superior to spring application. Rogler & Lorenz (155) reported that 90 pounds of nitrogen per acre supported 2271 pounds of dry forage per acre compared with 748 pounds without such fertilization. Fertilization of a heavily grazed pasture did more to improve it than 6 yr. of isolation from grazing.

A slowly available nitrogenous fertilizer can be formed from urea and formaldehyde, or urea and acetaldehyde [Scheffer *et al.* (161)]. Mruk *et al.* (126) observed that the urea-formaldehyde fertilizer released nitrogen at a rate adequate to support uniform growth of grass throughout a season. Warren *et al.* (199) found surprisingly little difference from year to year in the residual effect of ammonium sulfate and organic nitrogenous fertilizers.

Although ammonium salts are readily available to plants in general, they may be held rather strongly by base exchange materials in the soil. Losses of nitrate are normally much greater than losses of ammonia, although ammonia also can be lost in some quantity from sandy soils [McDowell & Smith (118)]. When aqua ammonia was used in irrigation water, Humbert & Ayres (92) found that volatilization accounted for up to 20 per cent of the total loss of nitrogen. In alkaline soils, the volatilization losses may exceed 50 per cent.

Jansson (97) has reviewed the literature and has reported extensive studies of the nitrogen transformations in soil; N^{15} was used as a tracer. He concluded that the supply of energy was the driving factor in nitrogen metabolism in soil with carbon and nitrogen transformations being completely interdependent. The bulk of ammonia fixation occurred immediately after its addition to soil, although some was later immobilized by incorporation into microorganisms. The period of binding in microorganisms was short, and this fraction usually accounted for only 10 to 15 per cent of the total soil organic nitrogen. By tracing with N^{15}, Jansson found that only one to four per cent of added fertilizer nitrogen was recovered in second and third oat crops. A part of the ammonia added to the soil was bound, whereas nitrate remained available; hence, processes using nitrate started quickly after its addition and a higher percentage of added nitrate than of ammonium nitrogen was taken up.

Ammonia is the preferred fertilizer for tea according to the observations of Gokhalé (76), although heavy applications resulted in some loss in tea quality. Gokhalé (77) concluded that ammonia was assimilated as such.

During a period of drought, Eck *et al.* (53) observed that anhydrous ammonia served as well as ammonium nitrate in the nutrition of wheat. Yields of grain did not increase appreciably although the protein content of the grain did. Anhydrous ammonia at about 150 pounds per acre also was effective for sugar beets in increasing the weight of beets, sugar and tops produced; 750 pounds per acre was not toxic.

Foliar diagnosis has been used as an index of the need for adding nitrogen. However, Balashev (6) has shown wide variations in nitrate in plants with time of day and among individual plants. Cook & Kishaba (45) found that the nitrate level in grape leaves exhibited a negative correlation with yield, and that addition of nitrogenous fertilizers to plants reasonably high in nitrogen depressed yields.

Foliar sprays of nitrogenous fertilizer, particularly urea, have been adopted with many plants and explored with others. Cain (31) observed that low levels of urea sprayed on leaves were almost entirely absorbed by coffee and cacao in 24 hr. and bananas in 30 hr. The lower surfaces of the leaves absorbed more rapidly than the upper, and young leaves absorbed more vigorously than old. Freiberg & Payne (68) found that as much as 65 per cent of urea applied to banana leaves was absorbed within 25 min. under humid conditions. Thorne & Watson (185) found that sprays of ammonium nitrate or urea increased the dry matter yield of sugar beet tops but not the roots; the yield of sugar was reduced somewhat. Kalinkevich (101) reported that the sulfhydryl content of lettuce leaves was greatly increased after spraying with urea.

Alteration of the appearance of fruits by nitrogen fertilization poses an acute problem to the producer. The poor color of apples produced with high nitrogen fertilization has been commonly observed. Schneider & McClung (162) reported that an increase in nitrogen fertilization decreased the color of peaches without influencing their size; in addition, maturation of the fruit was delayed. Jones *et al.* (99) found that high nitrogen was associated with the production of oranges with thick peels and low contents of juice and vitamin C. However, at too low a level of nitrogen, fruit production was reduced. High applications of nitrogen for a period of years suppressed the development of feeder roots and slowed the growth of orange trees [Ford *et al.* (62)].

The interaction of nitrogen with other elements in the soil and in the plant results in mutual alterations in the metabolism of the elements. Pleshkov (145) observed that high doses of phosphorus in the early stage of development of corn enhanced the rate of nitrogen metabolism, and good nitrogen fertilization in turn enhanced the uptake of P^{32} by the plants. Grunes *et al.* (81) also found that nitrogen fertilizers enhanced the uptake of phosphorus by barley and by sugar beets (80), a response associated with increased top and root growth. Ammonium ions antagonize magnesium ions, and Mulder (127) demonstrated that magnesium deficiency in plants increased as the nitrogen supplied was increased in its proportion of ammo-

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nium ion. Vlasyuk *et al.* (194) reported that nitrate enhanced the rate of sulfur metabolism in sugar beets as compared to ammonia nutrition. Viets *et al.* (189) observed that the uptake of zinc from the soil generally was decreased by fertilization with sodium nitrate and increased by the use of ammonium sulfate; the difference between the nitrogenous fertilizers was attributed to their influence on the soil pH.

Uptake and Transport of Nitrogen

Uptake and assimilation.—An expenditure of energy is required for the plant to take in ions from the external medium against a concentration gradient. Becking (8) has reviewed the literature on ion uptake and has conducted an extensive study of the uptake of ammonium ions in solution cultures of corn plants. The uptake of ammonium ions reached saturation at 10 p.p.m. and its half saturation point was 0.23 p.p.m. This indicated a very high affinity between ammonium ions and the carrier which transports it through the cell membrane. Hydrogen ions were released from the roots in an amount approximately equivalent to the ammonium ions taken up. Becking feels that cation uptake is linked to an active cation-induced respiration. Lack of nitrogen resulted in a marked depression in respiration. Stenlid (174) observed that the accumulation of nitrate by freshly excised wheat roots was increased by glucose and decreased by galactose. As the responses to chloride and nitrate were quite different, he concluded that each ion must be considered separately because of a difference in binding site and energy source functional in the absorption and accumulation of each ion.

Hattori (86, 87) has followed the uptake and assimilation of nitrogenous compounds by *Chlorella ellipsoidea*. He found that nitrogen-starved cells incorporated nitrogenous compounds in the following decreasing order: ammonia, arginine, urea, nitrate, ornithine, and citrulline. As soon as the nitrogen-starved cells accumulated some nitrogen, their rate of respiration increased. The assimilation of nitrogen and the increase in uptake of oxygen were suppressed by 2,4-dinitrophenol, azide, *p*-chloromercuribenzoate, carbon monoxide, cyanide, hydroxylamine, and other inhibitors of respiration or oxidative phosphorylation. Jensen (98) reported that C^{14}-phenylalanine and C^{14}-adenine were incorporated very slowly into the terminal 2 mm. of the root tips of *Allium cepa*. Cytoplasmic incorporation of adenine was much higher when division occurred, whereas incorporation of phenylalanine was unaffected; the reverse was true for nuclear and nucleolar incorporation. Bartholomew (7) has made extensive studies of the uptake of N^{15}-labeled nitrogenous compounds from soil, and Yemm & Folkes (207) recently reviewed their own work and that of others in this area.

Uptake and assimilation of nitrogenous compounds has been investigated in some detail with microorganisms [Gale & Folkes (72); MacMillan (115); Mandelstam (116); Cohen & Rickenberg (39)]. Cohen & Rickenberg presented the interesting concept that *E. coli* cells, which actively accumulate a variety of amino acids from the external medium, have a specific mechanism for concentrating each amino acid.

Excretion and mixed cropping.—Some years ago there was marked interest in the problem of nitrogen excretion from leguminous roots. Under conditions of low temperature, low light intensity, and long days such excretion does occur even from relatively young plants, and it will benefit the growth of associated nonleguminous plants. In most areas of the world, the nitrogen from legumes appears to be liberated by the breakdown of roots and nodules rather than by excretion from the intact root system. Nelson & Robins (130) reported that in a clover-grass mixture the clover supplied 99 pounds of nitrogen per acre to the grass when fertilizer was not applied. Walker *et al.* (196), on the other hand, found that clover utilized most of the N^{15}-labeled fixed nitrogen supplied to a grass and clover mixture. Frenzel (70) grew *Helianthus annuus* under aseptic conditions and observed the excretion of amino acids and amides from the intact roots. The total excretion was greatest under conditions optimal for growth. The root hair zone and the zone of elongation appeared to be the sites of excretion, and these two zones exhibited a specificity in the nitrogenous compounds they excreted. Frenzel concluded that the data were insufficient to establish whether the process concerned was an active excretion or merely a passive diffusion of the nitrogenous compounds.

Transport of nitrogen.—Ray *et al.* (151) have observed the movement of N^{15}-labeled ammonium nitrogen in soils. In Anoka loamy fine sand the movement of ammonia was mostly downward and lateral, whereas in Nicollet loam it was nearly symmetrical. The Nicollet loam retained much more of the ammonium nitrogen in the zone of injection. Kulaeva *et al.* (108) found that in pumpkin plants the amino nitrogen is transported mainly in the form of alanine, glutamic acid and γ-aminobutyric acid. Translocation in the nitrogen-fixing alder tree has been followed by Bond (16) with N^{15} as a tracer; the nitrogen normally is translocated in the xylem in the transpiration stream. Considerable N^{15} was detected in the shoot 6 hr. after exposing the nodulated roots to N^{15}-enriched molecular nitrogen. The mechanism of transfer of fixed nitrogen from the nodule into the transpiration stream was not apparent, for the alder nodule apparently is not a water absorbing organ.

Comparison of Nitrate and Ammonia in Plant Nutrition

Preference for the use of nitrate or ammonia in practical agriculture may be governed by a variety of factors—price, ease of application, pH of the soil, more vigorous binding and lower leaching of ammonia [Alexander (1)], drainage of the field, and the specific plant to be grown. However, experimental data indicate that under proper conditions plants usually assimilate ammonia more readily than nitrate. Rice is one of the most extensively studied plants in this regard and one of the earliest to reveal a clear superiority for ammonia. This observation again has been verified [Wahhab & Bhatti (195)]. Ozaki & Sasaki (139) demonstrated the rapid uptake of N^{15}-labeled ammonia by rice plants. After an hour, most of the N^{15} was in the nonprotein fraction of the roots and leaves, but in 6 hr. a higher proportion was assimilated into the protein fraction. With bacteria the uptake is

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much more rapid, and Burma & Burris (27) followed the kinetics of ammonia utilization by *Azotobacter vinelandii* with samplings as soon as one minute after the addition of N^{15}-labeled ammonia.

Other plants which have been reported to grow better on ammonia than on nitrate include the azalea [Colgrove and Roberts (42)], and *Epilobium angustifolium* [Tamm (182)]. Syrett (180) found that nitrogen-starved *Chlorella vulgaris* took up ammonia nitrogen about four times as rapidly as nitrate nitrogen at pH 6.1; both the soluble and insoluble organic nitrogen compounds increased much more rapidly in the ammonia fed cultures. Bershteĭn & Okaneko (9) reported a higher rubber content in the roots of kok-saghyz grown on ammonia rather than nitrate.

The uptake of nitrate and ammonia are considered to be pH dependent, with alkaline conditions favoring ammonia and acid conditions nitrate uptake. Wallace & Mueller (197) in a study of rough lemon cuttings found an average ratio of ammonia to nitrate absorption of 1.84. Ammonia absorption increased with rising pH, but nitrate absorption decreased with increasing pH only at the highest level of nitrate application; hence, they suggested that other published reports of pH effects on nitrate absorption might hold only for high application rates. Wander & Sites (198) also observed that nitrate absorption by rough lemon seedlings was relatively insensitive to pH. Thind & Duggal (184) found nitrites to be toxic to the fungus *Colletotrichum gloeosporoides* only under acid conditions; best utilization occurred at pH 8.

Although nitrate and ammonia may be used at different rates, the nitrate is reduced to ammonia before assimilation. It is not surprising that Vlasyuk *et al.* (194) observed that the amino acid composition of sugar beet plants is identical when grown on nitrate or ammonia. The result of Champigny (36) are more surprising, for she found a distinct difference in the balance of amino acids in hydrolysates of *Cholorella pyrenoidosa* cells grown on potassium nitrate or on urea; the composition of the soluble amino acid fractions from the two cultures were quite similar, however.

As pointed out over 20 years ago, plants accumulate a much higher level of organic acids when cultured on nitrate rather than on ammonia, and ammonia-fed plants are high in amides. Skvortsova (168) reported that *Nicotiana rustica* on nitrate accumulates 5.2 to 14.8 per cent malic plus citric acids as compared to 2.6 to 4.4 per cent when cultured on ammonia. Bershteĭn & Okaneko (9) also found an increased concentration of organic acids in nitrate-fed kok-saghyz.

Transformations among Nitrogenous Compounds

Nitrification.—Ammonia released from plant residues and ammonia added as fertilizer are subject to oxidation by the nitrifying bacteria; the nitrosomonas and nitrosococcus groups oxidize ammonia to nitrite and the nitrobacter group completes the oxidation to nitrate. The population of nitrifiers may drop to a low level in soils depleted of ammonia, for the organisms are autotrophs and dependent upon ammonia and nitrite oxida-

tion for energy. Organic matter does not favor their nitrifying activity in isolated cultures according to Kalinenko & Rozenberg (100), but Gundersen (82) has observed a marked stimulation of the early growth of *Nitrosomonas europaea* by a small amount of added corn steep liquor. The organisms are resistant in the soil, and when ammonia and water are provided, they develop rather rapidly [Calder (33)]. Under tropical conditions, nitrification is slow during the dry season but progresses rapidly when the rains return [Greenland (78)]. Ayres & Humbert (3) supplied ammonium hydroxide and ammonium sulfate to sugar cane plants and found that complete nitrification occurred in 20 to 85 days, depending upon the soil and the level of nitrogen supplied, but independent of the form in which the ammonium ions were supplied. Frederick (66) observed a 45-fold increase in the rate of nitrification at 27°C. as compared with 2°C. Frederick (67) concluded that the difference in the lag period before nitrification started in various soils was correlated with the initial population of the nitrifying organisms. Alexander (1) found that the ammonia level in fields in Australia receiving ammonia fertilizer in October was no higher than unfertilized controls by mid-January. Broadbent *et al.* (24) observed very rapid nitrification in soils low in ammonia and an inhibition by high levels of ammonia in poorly buffered soils. Excessively low as well as high pH and high salt concentrations in the soil also were inhibitory. In acid soils, a proper level of ammonia will adjust the pH to favor nitrification, a response observed in the laboratory by Eno & Blue (57).

There are a number of reports that the process of nitrification is accompanied by a loss in nitrogen. Dhar & Ghosh (51) indicated substantial losses from soil during nitrification of ammonia and urea, and attributed the loss to N_2 production from nitrite. Gerretson & de Hoop (75) found that losses of nitrogen oxides and N_2 were especially large (up to 74 per cent in pot experiments) below pH 5.5 and believed that the gases were formed from the reaction of nitrous acid with the ammonia supplied. No losses occurred under aseptic conditions.

Imshenetskiĭ & Ruban (93) reported that cell-free preparations from *N. europaea* oxidized ammonia and hydroxylamine to nitrite and indicated that hydroxylamine probably was a normal intermediate. Zavarzin (209) reported that addition of molybdate to molybdenum-starved cultures of *Nitrobacter* accelerated their oxidation of nitrite; riboflavin enhanced the effect of molybdenum. A wide variety of herbicides inhibited nitrification in soils in the laboratory [Otten *et al.* (138)].

Lees & Simpson (111) found that the oxidation of nitrite by *Nitrobacter* was inhibited by added chlorate (probably converted to chlorite) or cyanate, although cyanate stimulated the oxidation by dense suspensions of the organisms at a low pO_2 [Butt & Lees (30)]. The studies with inhibitors and by spectroscopic observations suggested to them that a cytochrome with an absorption maximum at 551 mμ (reduced) is intimately concerned with oxidation of nitrite by the organism. Butt & Lees (29) pointed out that the reduced bands at 521, 550, and 589 mμ were seen in cell suspensions

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in the presence but not in the absence of nitrite. In 50 volume per cent glycerol at the temperature of liquid air, the 589 mμ band was resolved into a 598 and a 580 mμ band. The *c*-type cytochrome was soluble, but the component absorbing at 598 mμ, presumed to be an *a*-type cytochrome, remained with a particulate fraction of the cells. The *c*-type cytochrome had an isoelectric point near pH 6.5, in contrast to the value of 10.65 reported for mammalian cytochrome-*c*. Although the authors did not speculate on the nature of the component absorbing at 580 mμ, the possibility that it is a hemoglobin is worth considering.

Denitrification.—Denitrification can be effected by a considerable variety of microorganisms; Sreenivasan (172) isolated 424 cultures from sea water and found that 55 per cent reduced nitrate and 8.7 per cent produced N_2. The process is an anaerobic one in which nitrate serves as the oxidant in place of molecular oxygen; Kefauver & Allison (103) have reported that oxygen and nitrite can be used simultaneously as oxidants by *Bacterium denitrificans*. With another organism, *Pseudomonas denitrificans*, Skerman & MacRae (167) observed no reduction of nitrate at an oxygen concentration above 0.2 p.p.m. They carefully measured the dissolved oxygen in their solutions and concluded that "nitrate reduction occurring in so-called 'aerated' solutions is due mainly to the activity of cells deprived of any oxygen supply." Measurement of the pO_2 above a culture or in the influent air gives a poor index of the oxygen actually present in a bacterial suspension; only measurements of dissolved oxygen, as used by Skerman and MacRae, approximate an accurate picture of the pO_2 at the surface of the cells. In fields rendered anaerobic by waterlogging or compaction, considerable losses of nitrogen may occur in the form of nitrogen oxides as well as N_2. The first step in denitrification, the conversion of nitrate to nitrite, is the same as the first step in the reduction of nitrate to ammonia, a step well defined in recent years (119). At this point, or after one more reducing step, the pathways diverge, although the two processes may occur simultaneously.

Nõmmik (135) has studied denitrification in soil with N^{15} as a tracer. Denitrification occurred only when the soil carried water in excess of 60 to 70 per cent of its water-holding capacity [also see (23)]. The concentration of nitrate had little influence on the rate of denitrification. As the nitrate level was increased, N_2O became the major reduction product. Denitrification was favored by a temperature of 15 to 20°C. [Bremner & Shaw (23), found that a much higher temperature was optimal] and by a relatively high pH. Reduction of N_2O was inhibited below pH 6, and at a low pH, NO was formed from nitrite. A good supply of readily oxidized organic material favored dentrification. Under the conditions found in cultivated soils, nonenzymatic dentrification did not occur. Studies of denitrification in the presence of $N_2{}^{15}O$ indicated that the nitrogen passed through the N_2O stage; apparently N_2O is normally a precursor of N_2 in the process, an observation also reported by Yamada & Virtanen (206). The N_2O and N_2 recovered did

not account for all the nitrate which disappeared. The microorganisms tested could not use N_2O as a source of nitrogen and the nitrifying bacteria could not oxidize it to nitrate. Mozen & Burris (125) have observed uptake of N_2O by *Azotobacter vinelandii*.

Loewenstein *et al.* (114) followed the loss of gaseous nitrogen from soil under greenhouse conditions. Soils left uncropped suffered large losses of nitrogen over a period of 11 months, up to 35 per cent in pH 5.5 soil amended with straw, and up to 72 per cent in soil at pH 6.5 without straw. In cropped soils, the losses were only 7 to 8 per cent. The greatest loss in nitrogen occurred during the first six weeks after fertilization. Bremner & Shaw (23) found that under conditions favorable for denitrification, 80 to 86 per cent of nitrate added to Rothamsted soil was lost in five days. De & Digar (49) also reported a considerably greater denitrification loss from ammonium sulfate or oil cake fertilizers added to uncropped soil as compared to soil fertilized four weeks after rice was transplanted to it.

The process of denitrification is inhibited by oxygen, for the oxygen and nitrate are competing oxidants. Collins (43) investigated the effect of aeration on denitrification by cultures of *Pseudomonas aeruginosa* and *Achromobacter* sp. in shaken flasks; vigorous aeration greatly decreased denitrification. Fedorov & Sergeyeva (59) have reported good growth of *Pseudomonas fluorescens* and *P. pyocyanaea* both aerobically and anaerobically in the presence of 0.001 *M* NaCN. Nitrate reduction was unaltered and yielded N_2, nitrites, and ammonia as products. Growth and denitrification were blocked by 0.005 to 0.01 *M* NaCN.

Iwasaki & Mori (95) observed that a washed suspension of denitrifying bacteria formed N_2 from nitrite and hydroxylamine in the presence of lactate. When lactate was absent, the organisms catalyzed a reaction between nitrite and hydroxylamine to yield primarily N_2O. Hyponitrite was not metabolized. A cell-free extract from the organisms formed gas, apparently N_2, from nitrite and dimethyl-*p*-phenylenediamine; Iwasaki and Mori postulated that nitrite is converted to hydroxylamine before reacting with additional nitrite to form the N_2. Bremner & Shaw (23) observed no formation of hydroxylamine during denitrification, although both nitrite and ammonia were demonstrated. Added hydroxylamine decomposed rapidly in soil, apparently by a purely chemical process.

Evans & McAuliffe (119, p. 189) have studied the nonenzymatic reduction of nitrite by ascorbic acid or DPNH. The products are NO, N_2O, and N_2 in the proportions 88, 3, and 9, respectively with ascorbic acid, and 75, 10, and 15, respectively with DPNH. The reaction with ascorbic acid occurs most rapidly at a low pH but is apparent at a pH as high as 6.

Nitrate reduction.—The main points of nitrate reduction were established in the few years preceding the period of this review, and the work is covered well by various authors in the symposium on *Inorganic Nitrogen Metabolism* edited by McElroy & Glass (119) and more recently by Nicholas (132) and Saris (159).

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Nason (119, p. 109) reviewed the enzymatic steps in nitrate reduction and indicated that highly purified nitrate reductases had been recovered from *Neurospora crassa* and from soybean leaves. The nitrate reductase from the neurospora has a marked specificity for TPNH, although DPNH will support reduction about 5 per cent as well. The enzyme from soybeans uses DPNH and TPNH equally well for nitrate reduction. FAD is the natural prosthetic group of the enzyme, although it can be replaced partially by FMN. Molybdenum also functions in the enzyme, and -SH groups are active. The equilibrium of the reaction lies so strongly toward nitrite that it has not been possible to demonstrate any formation of TPNH by reversal of the reaction. Nason has suggested the electron transport sequence TPNH $\rightarrow$FAD$\rightarrow$Mo$\rightarrow NO_3^-$ in the reduction process.

It has been reported frequently that light enhances nitrate reduction. A logical explanation for such observations is that light enhances the production of TPNH in the plant and the TPNH can serve as the electron donor for nitrate reduction. Bongers (18) investigated the role of light in the reduction of nitrate and nitrite and followed the kinetics of the process. He observed the liberation of 2 O_2 per molecule of nitrate and 1.5 O_2 per molecule of nitrite reduced to ammonia. In weak light there was a linear relationship between oxygen output and light intensity; at higher light intensities the dark reactions became rate-limiting. In the absence of suitable ammonia acceptors, ammonia was excreted into the medium. Bongers concluded that the quantum yield of O_2 evolved was the same for reduction of nitrate, nitrite, and carbon dioxide, but that the energetic efficiency of nitrate or nitrite reduction was much less than for photosynthetic reduction of carbon dioxide.

Kessler (104) observed that the green alga *Ankistrodesmus braunii* reduced nitrite rapidly in the light in an atmosphere of nitrogen devoid of carbon dioxide, although the organism reduced nitrate very slowly. Changes in temperature had little influence on the rate of nitrite reduction, as is characteristic of photochemical reactions. Cells adapted to liberate H_2 could use it for the reduction of nitrate. The characteristics of the reaction sequence suggested to Kessler that the reduction of nitrate to nitrite is dependent upon the production of photosynthate, whereas the reduction of nitrite is more directly affected by photochemical processes. Detailed tests by Bongers (17) with green algae led him to conclude that the external energy supply could be excluded as a rate-determining factor of the process of rebalancing the nitrogen and carbon of nitrogen-starved cells. The limiting factor for nitrogen assimilation in actively growing, as contrasted to nitrogen-starved cells, was the availability of suitable compounds to serve as nitrogen acceptors. The rate-limiting step in nitrate reduction appeared to fall between the reduction levels of nitrite and ammonia.

A number of investigators have implicated the cytochromes in nitrate reduction. Sato & Taniguchi *et al.* (119, pp. 87, 163) suggested that cytochrome-b_1 is in the electron transport chain of nitrate reduction in *Escherichia*

coli, and cytochromes-*c* and a_3 in *Micrococcus denitrificans*. Partial oxidation of cytochrome-b_1 was observed spectroscopically in *E. coli* when nitrate was added; the process was accompanied by the formation of nitrite. In a similar fashion, nitrate oxidized cytochome-*c* in *M. denitrificans* as evidenced by a decreased absorption at 550 mμ; changes in the absorption at 445 mμ suggested that an *a* type cytochrome also was involved, although no clear changes in the range 590–630 mμ were observed. In addition to the two types of nitrate reduction represented by *E. coli* and *M. denitrificans*, Sato believed that the strict anaerobes must reduce nitrate in still a third fashion. He indicated that one should make a distinction between "nitrate assimilation" (concerned with utilizing nitrate for synthesizing nitrogenous compounds in the cell) and "nitrate respiration" (concerned with nitrate as an oxidant in energy-yielding processes). Verhoeven & Takeda (119, p. 159) implicated cytochrome-*c* in the electron transport required for nitrate reduction by *Pseudomonas aeruginosa*. Although Egami *et al.* (54) found nitrate reductase in macerates of cotyledons from *Vigna sesquipedalis*, there was no nitrite reductase and no positive evidence for the participation of cytochrome-*b*.

Sadana & McElroy (158) purified nitrate reductase from *Achromobacter fischeri* and found that the reduced enzyme exhibited absorption peaks corresponding to cytochrome-*c*. Reduction of nitrate could be achieved with reduced benzyl viologen, or with DPNH in the presence of a bacterial DPNH-cytochrome reductase. Reduction by reduced benzyl viologen was not blocked by metal chelating agents, but reduction by DPNH was. Light reversibility of the CO inhibition of the system implicated the cytochrome system, and this was supported by spectral examination of the oxidation of reduced cytochrome by nitrate. Kinsky & McElroy (105) also have studied the properties of the nitrate reductase from *N. crassa*. The neurospora enzyme, purified seventyfold by ammonium sulfate fractionation, was stimulated by phosphate, a result attributed to its combining with the Mo of the enzyme to accelerate electron transport. The preparations carried a TPN-cytochrome-*c* reductase, and it was suggested that the flavin reductase catalyzed the rate limiting step for the overall system. Induction of nitrate reductase and cytochrome-*c* reductase occurred in parallel in the neurospora cultures grown on various levels of nitrate.

As mentioned, nitrate reductase is an adaptive enzyme in certain organisms. Tang & Wu (183) now have reported that rice seedlings grown in buffer alone or on ammonia did not have nitrate reductase activity, but that seedlings supplied nitrate elaborated the enzyme. The experiments were conducted in a fashion to eliminate interference by microorganisms. Candela *et al.* (34) found that the level of nitrate reductase always was low in cauliflower plants supplied ammonium sulfate.

Cheniae & Evans [(38)(119, p. 184)] always have found nitrate reductase in the nodules of leguminous plants, suggesting that if the enzyme is adaptive the substrate must normally be present in nodules. As the hemoglobin content of the nodules and the dry weights of the tops of soybeans were cor-

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related with the nitrate reductase activity of the nodules, they considered it reasonable to assume that nitrogen is fixed in an oxidative fashion to yield nitrates or nitrites which subsequently are reduced.

Cauliflower plants grown without added molybdenum were deficient in nitrate reductase [Candela *et al.* (34)]. Infiltration of molybdenum into deficient leaves was followed by an increase in nitrate reductase within 18 to 24 hr. Nitrate reductase activity decreased in plants held in the dark for two to six days but activity returned rapidly when the plants were illuminated. Takahashi & Nason (181) reported that tungstate inhibited the growth of *Azotobacter vinelandii* on nitrate by as much as 54 per cent, an inhibition which could be overcome with excess molybdate. The tungstate and molybdate operated in a competitive fashion.

Reduction of nitrite to ammonia.—The reduction of nitrate to nitrite has been studied intensively and detailed information on the process now is available, but the reactions for the conversion of nitrite to ammonia are less clear. Nason (119, p. 109) and McElroy & Spencer (119, p. 137) have reviewed the usually accepted series of reductions through hydroxylamine to ammonia, and Steinberg (119, p. 153) has discussed his experiments with nitrohydroxylaminate. Although this compound was utilized by his test organisms, there was no evidence that it was reduced through the stages of hyponitrite and hydroxylamine. Fedorov & Il'ina (58) reported that soil actinomycetes reduced nitrate and nitrite readily and suggested that hydroxylamine and ammonia were intermediates because of their ready assimilation. McNall & Atkinson (120) indicated that their trained strain Bn of *E. coli* in addition to growing on nitrate or nitrite as a sole source of nitrogen, also could utilize hyponitrite, hydroxylamine, or N_2O. The slow adaptation to N_2O indicated that it probably was not an intermediate in nitrate reduction. Strain Bn, but not the parental strain, was reported to reduce nitrite, hyponitrite, and hydroxylamine to ammonia in an atmosphere of hydrogen. In common with most experiments of this type these tests left a real question as to whether the intermediates supplied were used as such or were assimilated only after their spontaneous breakdown. The prolonged lag in growth often exhibited by the cultures was not reassuring on this point.

Spencer *et al.* (171) studied a soluble nitrite and hydroxylamine reductase system from *Azotobacter agile*, and found that reduced pyridine nucleotides served as the electron donors. Added flavins enhanced the activity, FAD being the active compound with nitrite reductase and FAD or FMN with hydroxylamine reductase. Both the nitrite and hydroxylamine reductases are adaptive enzymes and as their formation is stimulated by nitrate but not by N_2, ammonium sulfate, or glutamate, it appears that the enzymes are functional in nitrate and nitrite assimilation but not in nitrogen fixation. Medina & Nicholas (121) examined the reduction of nitrite by *N. crassa* and indicated that metallo-flavoprotein enzymes were responsible for the reduction of nitrite, hyponitrite, and hydroxylamine. Nicholas (131) has reviewed pertinent recent work on this subject from his own and other laboratories. Iron and copper activated the nitrite and hyponitrite reductases, and manganese

activated the hydroxylamine reductase, as observed by Spencer *et al.* (171). Their different responses to zinc deficiency indicated that nitrite and hyponitrite reductases were separate enzymes. Roberts & Azim (119, p. 176) reported that use of nitrite by *A. vinelandii* was preceded by a lag period, and contrary to the general experience of others, they observed a lag in the use of ammonia at concentrations above 1×10^{-4} *M*. De Turk & Bernheim (50) described a mycobacterium which assimilated hydroxylamine. Grossowicz & Lichtenstein (79) selected a strain of *E. coli* which would catalyze the disappearance of hydroxylamine, and found that the reaction produced no nitrate, nitrite, ammonia, N_2O, or N_2; aspartate was the suggested product. Cell-free preparations from the organism were active only when fumarate or malate was added.

Pichinoty & Senez (143) reported that *Desulfovibrio desulfuricans* can reduce nitrite or hydroxylamine to ammonia. Upon treatment with hexadecyltrimethylammonium bromide the organisms lost this ability, but after washing, the cells regained it when cytochrome-c_3 was added. The reactivation of hydroxylamine reductase was especially marked. Thus, a role in nitrite and hydroxylamine reduction was assigned to cytochrome-c_3, the first cytochrome pigment to be isolated from anaerobic organisms. Pyruvate served as hydrogen donor *via* cytochrome-c_3 for the reduction of nitrite to ammonia (144). In a continuation of their work on *D. desulfuricans*, Senez & Pichinoty (165) found that the organism reduced hydroxylamine to ammonia with H_2 as the reducing compound. Nitrite inhibited the reduction of hydroxylamine as did heavy metals, oxidizing agents, cyanide, and phenylhydrazine; heavy metals were not implicated in the catalysis. In the next fascinating chapter of the story, Senez & Pichinoty (166) reported that actual reduction of hydroxylamine occurred by a nonspecific, nonenzymatic process. Any agent (H_2, pyruvate, etc.) which reduced cytochrome-c_3, with the appropriate enzyme, supported hydroxylamine reduction, for the reduced cytochrome-c_3 in the absence of any enzyme reduced hydroxylamine to ammonia. Reduced benzyl viologen or beef heart ferrocytochrome-*c* also were chemically reoxidized by hydroxylamine, although they reacted less rapidly than cytochrome-c_3. Senez and Pichinoty suggested that any organism possessing hydrogenase has the potentiality for hydroxylamine reduction. This concept may provide a key in the explanation of the association of hydrogenase with nitrogen-fixing organisms. If hydroxylamine is a normal intermediate in nitrogen fixation, the hydrogenase may serve to reduce cytochrome (or another compound with similar function) which in turn will reduce hydroxylamine to ammonia.

Virtanen & Saris (193) observed the formation of organic hydroxylamine compounds by *Torulopsis utilis*. A heavy suspension of the organisms was supplied with a nitrite solution, and after incubation, the supernatant recovered carried 240 μg. of bound hydroxylamine nitrogen in 1150 ml. from the 86 mg. of nitrite nitrogen supplied initially, or a 0.28 per cent conversion of the nitrogen supplied. After reducing the bound hydroxylamine fraction with sodium amalgam, the appearance of glutamic acid, aspartic acid, and

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glycine indicated that oximes of pyruvic, α-ketoglutaric, and oxalacetic acid had been present. Virtanen & Saris suggested that the physiological function of hydroxylamine binding may be to trap hydroxylamine which is not reduced quickly to ammonia. Saris & Virtanen (160) also followed the production of bound hydroxylamine by *Azotobacter chroococcum*. The hydroxylamine appeared to be bound to amino acids which had been incorporated into polypeptides, although the low concentration of bound hydroxylamine in the isolated fractions left considerable question regarding this point.

Hyponitrite has proved elusive as an intermediate. Medina & Nicholas (122) observed a hyponitrite reductase in *N. crassa*. As the enzyme produced ammonia when incubated with hyponitrite and DPNH, they suggested that hyponitrite is the intermediate between nitrite and hydroxylamine in nitrate reduction. McNall & Atkinson (120) also reported reduction of hyponitrite by their strain of *E. coli*. Frear & Burrell (65) synthesized hyponitrite with an N^{15} label and infiltrated it into soybean leaves. N^{15} analysis indicated that it was oxidized to nitrite or nitrate before reduction to ammonia, so the tests were inconclusive for its role in nitrate reduction.

Hydrazine has attracted little attention as an intermediate in nitrogen metabolism, perhaps because of its reactivity and toxicity, because its two nitrogen atoms require the formation of an N—N bond, and because it does not fit the reduction of nitrate by two electron steps. Riggio-Bevilacqua (153) reported a hydrazine dehydrogenase in extracts from seedlings of *Pisum sativum*, but found (154) that hydrazine strongly inhibited the growth of *Cucurbita pepo*. Bach (4) also has presented evidence suggesting that hydrazine may be formed by nitrogen-fixing organisms and may react with α-ketoglutaric acid to form a cyclic compound. In this connection it is interesting to note the recent announcement by a commercial firm that they will produce glutamine by reacting hydrazine with α-ketoglutaric acid to form a cyclic intermediate which they will open by hydrogenation.

Metabolism of Nitrogenous Compounds

Transamination.—Transamination is a general reaction in plants and undoubtedly is very important in the synthesis of most of the amino acids found in plants. Little has been added during the past two years to extend this basic conclusion. Jacobi (96) has surveyed a variety of transaminases of *Ulva lactuca* and again has observed the remarkable activity of the glutamate-aspartate transaminase. Cook (44) has followed transamination in corn radicles and has calculated reaction rates with corrections for the changes in substrate concentration occurring during the reaction.

There has been considerable recent work on inhibitors of transamination. Garcia-Hernández & Kun (73) found that hydroxyaspartic acid inhibits competitively in the transamination of aspartate to α-ketoglutarate. This reaction also was inhibited noncompetitively by diaminopropionate and with mixed competitive and noncompetitive inhibition by diaminosuccinate. Das & Rao (48) reported that benzyl-, isobutyl- and allyl-isothiocyanates inhibited the glutamic-alanine transaminase. Braunshteĭn & Azarkh (20)

observed that tissue transaminase was decreased in rats on a diet deficient in vitamin B_6 when isonicotinoyl hydrazide was administered. Hicks & Cymerman-Craig (90) found that isonicotinic acid hydrazide, nicotinic acid hydrazide, picolinic acid hydrazide, benzohydrazide, and isonicotinic acid hydrazide methiodide at a concentration of 3.3×10^{-2} *M* gave about 50 per cent inhibition of glutamic acid production by the alanine-glutamate transaminase from pig heart. The action of these hydrazides was attributed to their modification of the coenzyme so that it could not combine with the substrate.

Production of amino acids.—The conversion of ammonia to organically bound nitrogen is dependent chiefly on glutamic acid dehydrogenase. The formation of aspartic acid by the action of aspartase on fumaric acid and ammonia serves as an alternate means of incorporating ammonia. Ellfolk (55) has published an extensive review on the nature and activity of aspartase.

Radhakrishnan & Meister (149) have demonstrated that a purified preparation of amino acid oxidase, incubated anaerobically with an amino acid and an α-keto acid, produced an amino acid corresponding to the α-keto acid supplied. For example, D-amino acid oxidase plus pyruvate and D-phenylalanine produced D-alanine. The reaction depended upon the presence of FAD. Although the overall reaction appears the same as a transamination, the reaction does not involve amino group transfer but requires the intermediate participation of ammonia. Zelitch (211) has observed the same type of reaction with crude preparations from plant material, and Wiame & Piérard (202) have reported L(+)-alanine dehydrogenase in *Bacillus subtilis*.

The review by Kretovich (107) on amides and the dicarboxylic amino acids is of special interest, because it offers in English a description of his experimental results complete with data. Preparations from rye and pea seedlings oxidized glutamic acid most rapidly among the amino acids. Aspartic acid also was oxidized rapidly, and the oxidation of these dicarboxylic amino acids appeared to be stimulated by light. Kretovich indicated that phenolic compounds are active in amino acid oxidations by sunflower seedlings. It is assumed that glutamic acid normally is formed in plants by reductive amination, but the experiments of Kretovich are among the few which support this assumption directly. Reductive amination of oxalacetate to form aspartate is much less clearly established, although work from the laboratory of Kretovich indicated that homogenates of pea seedlings catalyzed such a reaction as well as a synthesis by aspartase. Kretovich, however, feels less reservation about alanine synthesis by reductive amination and has stated "plants exhibit an intense process of direct amination of pyruvate by ammonia." His experiments on alanine synthesis were performed some years ago with dialyzed preparations from pumpkin seedlings. Wiame & Piérard (202) have demonstrated the reversibility of the DPN-linked L-alanine dehydrogenase in *B. subtilis*.

Kretovich (107) also has been interested in the biosynthesis and transformations of glutamine and asparagine in plants. His group checked the

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incorporation of N^{15} into the amide and amino groups of glutamine (sugar beets) and asparagine (etiolated lupine and vetch seedlings). N^{15} was incorporated more rapidly into glutamine than into asparagine, and the amide groups were more heavily labeled than the amino groups.

Rohrlich & Rasmus (156) studied the glutamic acid decarboxylase of wheat and rye germ and followed its production of γ-aminobutyric acid. The reaction required pyridoxal phosphate. Naylor & Tolbert (129) observed that the same reaction was catalyzed in barley leaves; γ-aminobutyric acid and carbon dioxide were the only detectable products of the action of glutamic acid decarboxylase on labeled glutamic acid.

Bilinski & McConnell (10, 11) have investigated the synthesis of amino acids by wheat plants supplied C^{14}-labeled acetate. Glutamic acid, proline, and arginine accumulated the highest levels of C^{14}. Acetate-2-C^{14} usually gave more highly radioactive amino acids than did the carboxyl-labeled acetate. Eighty-three per cent of the C^{14} incorporated from acetate-1-C^{14} into glutamic acid was found in its carboxyl groups. The total data suggested that the tricarboxylic acid cycle provided a major pathway for biosynthesis of the dicarboxylic amino acids.

Toxicity of nitrogenous compounds.—Plants may be seriously damaged by fertilization with urea carrying biuret as a contaminant. Webster *et al.* (201) found that biuret was essentially without effect on absorption or hydrolysis of urea, oxidation of amino acids, decarboxylation of glutamate, hydrolysis of arginine, and breakdown of leaf protein by *Xanthium* leaves. Its only observed toxicity was exhibited in a decrease in total leaf proteins and a blocking of incorporation of amino acids into proteins. Drosdoff *et al.* (52) applied urea annually to tung trees, but did not observe necrotic spots on the leaves until the fourth year. The toxic agent, possibly biuret, appeared relatively immobile, for the necrosis was observed only on the leaves near the base of the shoots. Further studies on biuret toxicity showed that 2.5 per cent of biuret in urea inhibited the germination of wheat up to 30 per cent when the urea was applied in intimate contact with the seeds at levels of 7.5 pounds per acre or greater; urea itself was innocuous [Smika & Smith (169)]. Funabiki *et al.* (71), however, found no influence of biuret, at much lower concentrations, on barley germination. Biuret at 15 to 20 p.p.m. inhibited the growth of barley in sand cultures. It also inhibited nitrification and was decomposed very slowly in soil. Rotini (157) voiced disagreement with the accepted toxicity of biuret and suggested that cyanic acid formed from urea in the soil is responsible for the observed toxicity. His explanation does not reveal why purified urea is nontoxic when compared to urea contaminated with biuret.

Plant Composition

Amino acids.—Kasting & Delwiche (102) observed the amino acids of the ornithine cycle in wheat, barley, and watermelon seedlings. In the wheat and barley, citrulline and ornithine were present in only one to two per

cent of the concentration of arginine, but the levels of the three compounds were approximately equal in watermelon seedlings. Coleman (41) found ornithine and citrulline (40) in sulfur-deficient flax and isolated and characterized the compounds. On the basis of this work and that of others, Coleman has suggested that the ornithine cycle occurs in plants, and that under sulfur deficiency, disruption of arginine metabolism may lead to accumulation of ornithine.

Fowden & Webb (64) studied the entry of C^{14} from $C^{14}O_2$ into amino acids in the leaves of peanut plants. γ-Methyleneglutamic acid and γ-methyleneglutamine accumulated little C^{14} and had no apparent influence on photosynthesis. The main site for synthesis of γ-methyleneglutamic acid was the cotyledons. Fowden & Steward (63) and Zacharius *et al.* (208) have been interested in the metabolism of some of the newly described nitrogenous compounds in plants. The occurrence of such compounds as γ-methyleneglutamine and azetidine-2-carboxylic acid suggests the occurrence of unrecognized metabolic pathways in plants. In the genus *Tulipa* they found that γ-methyleneglutamine varied from 60 to 70 per cent of the free amino acid nitrogen down to levels too low to detect. Mature leaves on excised shoots of *T. gesneriana* did not actively incorporate C^{14} from $C^{14}O_2$ into γ-methyleneglutamine; the compound did not appear to fill the usual role of glutamine. Floral initiation and development in the tulip was accompanied by extensive changes in the soluble nitrogen fraction; arginine decreased and glutamine and asparagine became prominent. However, γ-methyleneglutamine and γ-methyleneglutamic acid did not participate appreciably in these changes.

Paech (140) grew winter wheat seedlings on various levels of urea, and found more carotenoid formation in cultures given the lower concentrations of urea. Apparently when abundant nitrogen was available, the synthesis of protein was favored over non-nitrogenous compounds. Tso & Jeffery (186) found that tobacco scions on tomato stock produced much more nicotine than was expected from earlier reports by others. The alkaloids in these grafted plants carried about 6 atom per cent N^{15} excess after four weeks' culture on nitrate containing 20 atom per cent N^{15} excess. Nornicotine produced in the roots of plants composed of tomato scions on tobacco stock contained a higher concentration of N^{15} than did nicotine; this indicates that the synthesis of nornicotine is independent of nicotine synthesis.

Virtanen (190) has reviewed the results of the search by his laboratory group for new amino acids in plants. This reference (190), together with that of Steward & Pollard (176) constitute a comprehensive review of this subject. Virtanen & Ettala (191) have found that dihydroxyglutamic acid is rather common as a free amino acid in plants. They observed it in 26 species including flowering plants, a fern, a moss, and mushrooms. Burroughs (28) isolated and characterized 1-amino-cyclopropane-1-carboxylic acid from pears and apples. Vähätalo & Virtanen (188) obtained the same compound from cowberries, and observed bound homoserine in cowberries and

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cranberries. Matikkala & Virtanen (117) recovered a new sulfur-containing amino acid from onions but only partial characterization was reported. Lioret (112) examined the alcohol extracts of a variety of crowngall tumor tissues and identified a total of twenty-one free amino acids. Two unknown compounds, reacting as amino acids, also were observed, and one of these was recovered in a crystalline state but was only partially characterized. Among the free amino acids of alfalfa hay recovered by ethanol extraction, Coulson (47) found pipecolic acid, baikiain, citrulline, and either ϵ-aminohydroxycaproic acid or methionine sulfone. Certain unknown compounds observed were tentatively identified as α, ϵ-diaminopimelic acid or ethanolamine phosphate, and 3,4-dihydroxyphenylalanine.

Ogino (136) observed that the main nonprotein nitrogenous compounds of marine and fresh water algae were free amino acids. In the marine algae the free monoamino acids were dominant, whereas in the fresh water algae the basic amino acids were most abundant. The fresh water alga *Prasiola japonica* had as much as 2.6 per cent of its dry weight as free arginine.

The free amino acid contents of nineteen species of the euphorbia were remarkably similar [Montant (124)]. Alcoholic extracts of stems and leaves yielded seventeen common amino acids; aspartic acid was dominant in the stems and glutamic acid in the leaves. Two unidentified compounds yielded ninhydrin positive spots on the paper chromatograms. Menoret (123) separated the upper portion of carrot roots into phloem, cambium and medullary parenchyma and determined the free amino acids in each fraction. The tissues were distinctly different in the amount and distribution of the amino acids they carried. The cambium had the highest concentration of nitrogenous compounds, including much glutamine and arginine. The other amino acids generally were most concentrated in the phloem. Carrot tissue cultures were poorer in free amino nitrogen than the fresh tissues, and the amino acid composition differed from that of the fresh tissues. Zsoldos (212) found that when the supply of ammonia was withdrawn from rice plants, the total amino acids, asparagine, glutamine, and peptides remained virtually constant for 17 hr., while valine and leucine declined. When the supply of ammonia was increased, histidine, aspartic acid, and leucine remained unchanged in the roots, valine and alanine and glutamine increased sharply, and tyrosine disappeared. In the experiments of Pleshkov *et al.* (146), the free amino nitrogen in kidney bean leaves declined under nitrogen deficiency and increased somewhat with phosphorus and potassium deficiencies. The variation in arginine content was the most sensitive index observed for judging the nutritional state of the plants.

Control of the amino acid composition of a crop as widely used as wheat could have a great impact on the nutrition of a large segment of the world's population. The results of Lawrence *et al.* (109) hold promise for a breeding program to improve wheat protein, for their analysis of 286 samples of wheat and related plants showed a variation in lysine content from 2.46 to 3.84 per cent. The mean lysine content was 2.89 per cent and for fifteen hybrid selec-

tions 3.10 per cent. Gunthardt & McGinnis (83) found that although they could increase the protein content of wheat by adequate nitrogen fertilization, there was little change in the amino acid composition of the protein; the high protein samples were somewhat lower in concentration of lysine.

Stein v. Kamienski (173) separated 36 primary, secondary, and tertiary amines and diamines from plants by paper chromatography with two solvent systems. Stein's survey for steam volatile amines included 220 species of flowering plants and mosses, and in a sample of 75 of these, 25 species had methylamine, 1 had dimethylamine, 15 had isobutylamine, 75 had isoamylamine and 16 had β-phenylethylamine.

Nitrogenous compounds in plant sap.—Bakhuis (5) identified homoserine (observed earlier in pea plants by Virtanen's group) in the sap of inoculated pea plants in addition to aspartic acid, asparagine, and glutamine. Bollard (14, 15) has conducted a detailed study of the nitrogenous compounds in sap. He found nitrate in the xylem sap of fewer than half the species of dicotyledons, monocotyledons, and gymnosperms examined. Glutamine or asparagine normally was quantitatively dominant, with glutamine holding this position about three times as frequently as asparagine. Citrulline was found in the sap from 29 families but was not detectable in any monocotyledon. Allantoin and/or allantoic acid were present in the sap from 23 families, and in some species allantoic acid was a major constituent. Organic compounds accounted for most of the nitrogen in the tracheal sap of apple trees, and a peptide was present with the free amino acids. Aspartic acid, asparagine and glutamine were dominant, and the nitrogenous composition of the sap was little affected by the variety of apple, the root stock, or the fertilizer treatment. The soluble nitrogen fraction from leaves and fruits was quite different from that of tracheal sap. The nitrogen of the tracheal sap of pear, quince, plum, peach, apricot, and cherry, as in the apple, was chiefly in the form of organic compounds.

Influence of other nutrients on nitrogenous compounds.—Possingham (147, 148) has investigated the influence of mineral nutrition on the free nitrogenous compounds in tomato plants and Yemm & Folkes (207) have discussed his work. Deficiencies of zinc, copper, manganese, or iron induced increases in the free amino acids of the plants. Iron and zinc deficiencies caused large increases in asparagine and glutamine. In contrast to the other deficiencies, molybdenum deficiency reduced the level of soluble nitrogenous compounds. Each deficiency gave a characteristic amino acid spectrum, but the differences were not sufficiently distinctive for diagnostic purposes. Responses to added nutrient elements sometimes were rapid, *e.g.*, within 4 hr. after the addition of molybdenum to deficient plants, appreciable changes in the concentrations of individual amino acids occurred. Hewitt *et al.* (89) also have studied the influence of molybdenum on nitrogen metabolism. They found that the total nitrogen content of cauliflower plants increased when plants supplied nitrate were furnished adequate molybdenum. Molybdenum-deficient plants supplied ammonia usually had as much protein,

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α-amino nitrogen, and total organic nitrogen as plants grown with nitrate and adequate molybdenum. Plants grown with ammonia or urea had high levels of arginine, and molybdenum deficiency increased this amino acid.

Coleman (40) has investigated the influence of sulfur deficiency on the free amino acid balance in white clover, flax, *Desmodium uncinatum*, tomato, and barley. The level of free arginine increased greatly in sulfur-deficient *D. uncinatum*, white clover, tomato, and flax, while the glutamic acid level decreased (except in flax) and amides accumulated. Asparagine was the dominant amide in sulfur-deficient legumes, whereas glutamine was more abundant in the tomato. Glycine and serine increased in all the sulfur-deficient species. Mulder & Bakema (128) have compared the free amino acid and protein balance of normal and deficient potato tubers. The amino acid composition of the protein was independent of the supply of nitrogen, phosphorus, or potassium, although the protein varied in amount. The balance among the free amino acids, in contrast, was responsive to changes in the mineral nutrition; abundant nitrogen or limited phosphorus or potassium increased the soluble nitrogen and the proportion of amides in the nonprotein fraction. Schütte & Schendel (163) grew bush beans in nutrient solutions deficient in micronutrient elements and determined the amino acids in hydrolysates of the entire plant protein. Copper-deficient plants produced a protein high in threonine and low in valine, and zinc-deficient plants a protein low in glycine and isoleucine.

Disease and plant composition.—Disease may alter the nitrogen metabolism of plants; for example, Henke (88) found that in potato plants infected with leaf roll disease the nitrogen content of young tubers was much greater than in tubers of normal plants, although the nitrogen of the whole plant was decreased. Reindel & Bienenfeld (152) found that the same free amino acids were present in healthy potato plants as in those infected with leaf roll virus, but that glutamic acid and valine were higher in the juice from infected plants. Fife (60) observed three times the normal level of arginine in the juice expressed from the leaves of sugar beets infected with curly top virus, and the average level of all the free amino acids was about twice as high in the diseased as in the normal leaves. Amino acid analogues can influence plant differentiation [Hotta & Osawa (91)] and it is possible that an abnormal balance of amino acids also may influence plant development. Virtanen & Linkola (192) observed abnormalities in the form of pea plants supplied α-alanine, β-alanine or phenylethylamine under conditions of culture which left the root system aseptic. Army & McNeal (2) found that symptoms of barley stripe mosaic in wheat were generally increased in eight varieties of wheat when fertilized with nitrogen. Several isolates of verticillium wilt organisms induced wilt more rapidly in *Antirrhinum magjusgrandiflorum* plants growing with normal or excessive nitrogen than in plants on nitrogen deficient soil [Isaac (94)], and isolates of *Verticillium nublilum* and *V. tricorpus* produced a greater incidence of wilt in plants growing on a soil supplied with heavy dressings of an organic nitrogenous fertilizer than in plants

on a nitrogen-deficient soil. Selman & Grant (164) observed that increasing the supply of nitrogen above the optimal level for growth of tomato plants increased their susceptibility to systemic infection by tomato spotted wilt virus and increased the virus content of the plants. Zeevaart (210) found that the free amino acid content of *Lupinus luteus* plants was much lower in plants inoculated with *Fusarium oxysporum* than in uninoculated plants. He assumed that the fusarium lives on amino acids as a carbon source in the xylem vessels both of the resistant and susceptible plants.

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METABOLISM OF CARBON COMPOUNDS[1,2]

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This review will be restricted to an evaluation of the investigations concerned with the metabolism of carbohydrates and related compounds in plants. No distinction will be made in this review between the higher and lower forms of plants. In addition, reference will also be made to animal tissues since most biochemical pathways which are pertinent to this review appear to be similar in animal and plant tissues.

For convenience, this review is organized around the framework of the major pathways. The reactions converting other carbohydrates and related compounds to intermediates common to a major system are discussed as subdivisions of the main routes. The major pathways are the Embden-Meyerhof-Parnas (EMP) pathway, the pentose phosphate pathway, and the citric acid cycle.

The literature published since the review of Axelrod & Beevers (1) in Volume 7 of this series will receive primary attention. In the allotted pages, it is not possible to give credit to all the papers pertinent to this field published since November 1955. The writer hopes that in their reviews his colleagues will forgive his lack of good judgment.

The Embden-Meyerhof-Parnas Pathway

This pathway, historically, was developed to account for the anaerobic breakdown of glucose to ethanol and carbon dioxide by yeast and the anaerobic degradation of glycogen to lactic acid by mammalian muscle. However, as more organisms were investigated, it became evident that this pathway had a more general application and that ethanol, CO_2 and lactic acid could be considered as special cases. Many authors, including this reviewer, consider the pathway to terminate at pyruvic acid. That the EMP pathway catalyzing the conversion of starch and glucose to pyruvic acid exists in plants has been well documented by the reviews of Stumpf (2, 3) and Axelrod & Beevers (1). Evidence for an EMP pathway from glucose to

[1] The survey of literature pertaining to this review was concluded in October 1958.

[2] The following abbreviations will be used: ADP (adenosine diphosphate); ATP (adenosine triphosphate); CoA (coenzyme A); 2,4-D (2,4-dichlorophenoxyacetic acid); DPN (diphosphopyridine nucleotide); DPNH (reduced diphosphopyridine nucleotide); EMP (Embden-Meyerhof-Parnas pathway); GDP (guanosine diphosphate); GTP (guanosine triphosphate); HMP (hexose monophosphate pathway); K (equilibrium constant); KDPH (2-keto-3-deoxy-7-phosphoglucoheptonic acid); P_i (orthophosphate); PP (pyrophosphate); TPN (triphosphopyridine nucleotide); TPP (thiamine pyrophosphate); TPNH (reduced triphosphopyridine nucleotide); UDP (uridine diphosphate); UDPG (uridine diphosphoglucose); UDPGal (uridine diphosphogalactose); UTP (uridine triphosphate).

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pyruvic acid (ethanol and CO_2) was based on: (*a*) formation and utilization of postulated intermediates; (*b*) presence of enzymes catalyzing postulated reactions of the EMP pathway; (*c*) sensitivity of these enzymes to the inhibitors, iodoacetic acid and fluoride; and (*d*) dissimilation of specifically labeled glucose to products labeled as predicted by the EMP pathway, i.e., glucose-1-C^{14} to methyl-labeled ethanol and glucose-3,4-C^{14} to unlabeled ethanol and $C^{14}O_2$. A recent striking confirmation of the first three pieces of evidence is furnished by Hatch & Turner (4) with an extract of pea seeds, while Beevers & Gibbs (5) have provided the confirming tracer data.

Tracer evidence.—The specifically labeled glucose-C^{14} technique which has been used successfully to determine the pathway of glucose dissimilation in bacteria [Gunsalus & Gibbs (6)] and plants (5) has been applied to the golden brown alga, *Ochromonas malhamensis* by Reazin (7). This organism ferments glucose to 1.6 moles of CO_2, 1.75 moles of ethanol, and 0.18 moles of lactic acid. Glucose-1-C^{14} and glucose-6-C^{14} yielded methyl-labeled ethanol. In the presence of arsenite, the pyruvic acid which accumulated from glucose-2-C^{14} was labeled in the α-carbon whereas that from glucose-1-C^{14} was methyl labeled. It was concluded that the EMP pathway is the pathway of glucose dissimilation by this alga under both aerobic and anaerobic conditions.

Phosphorylase.—In 1949, Arreguin-Lozano & Bonner (8) observed that ethanol extracts of potatoes which have been held at 25°C. depressed the formation of iodine-staining polysaccharides in partially purified digests. They concluded that these extracts contained a potent, specific phosphorylase inhibitor. Schwimmer (9) has investigated the nature of the inhibitor. He has found that it is related to chlorogenic acid and that apparently most of the inhibitor activity is due to its caffeic acid moiety, since caffeic acid but not quinic acid inhibits. Obata *et al.* (10, 11) have reported the inhibitor to be in the saponin fraction of the roots of *Beta vulgaris* and *Beta vulgaris crassa* (mangold), as well as the bark of *Quillaja sponaria.* No further purification of the saponin fraction has been reported by the Japanese group.

Hexokinase.—Medina & Sols (12) have confirmed the observation of Saltman (13) that pea seed homgenates contain a particulate hexokinase which phosphorylates glucose, fructose, and 2-deoxyglucose. N-Acetylglucosamine is a competitive inhibitor of each of the three substrates. Medina & Nicholas (14) have made the observation that the hexokinase activity of *Neurospora crassa* grown in a Zn^{++} deficient medium is decreased. Hexokinase activity was regained within 12 hr. when Zn^{++} was added back to the growth medium. They also noted than Zn^{++} added *in vitro* was moderately effective in protecting the *Neurospora* hexokinase from inactivation by *p*-chloromercuribenzoate. Williams *et al.* (15) have implicated a hexokinase as the site of biotin involvement in *Saccharomyces cerevisiae.*

The $\Delta F°$ of hydrolysis of ATP has been determined by making use of the

equilibrium constant (K) of the hexokinase reaction. The reactions involved are:

$$\text{ATP} + \text{glucose} \rightleftharpoons \text{glucose-6-phosphate} + \text{ADP} \qquad \Delta F_1 \qquad 1.$$

$$\text{glucose-6-phosphate} + H_2O \rightleftharpoons \text{glucose} + P_i \qquad \Delta F_2 \qquad 2.$$

the summation of which gives

$$\text{ATP} + H_2O \rightleftharpoons \text{ADP} + P_i \qquad \Delta F_3 \qquad 3.$$

Robbins & Boyer (16) obtained the K of equation 1 by measuring the incorporation of glucose-C^{14}-6-phosphate into glucose, while Vladimirov *et al.* (17) employed glucose-6-phosphate labeled with P^{32} in the same way. Using the relationship $\Delta F° = -RTLnK$, free energy values for the hexokinase reaction at pH 7.0 were calculated to be -4.5 and -4.7 kcal. for zero (catalytic) and excess Mg^{++} concentrations by Robbins & Boyer, while the Russian group reported -2.6 to -4.4 values for the hexokinase reaction at pH 7.25. While one group (16) used a $\Delta F°$ of -3.1 kcal. for equation 2, the other (17) quoted a value of -2.45 which, combined with the $\Delta F°$ of equation 1, gives a $\Delta F°$ for the hydrolysis of ATP of -7.6 to -7.8 kcal. for Robbins & Boyer and -5.05 to -6.85 kcal. for Vladimirov *et al.*

Phosphoglucomutase.—While this enzyme was found to be widely distributed in higher plants and especially rich in broad bean seeds by Morita *et al.* (18), its absence was reported in sea lettuce, *Ulva lactuca*, by Jacobi (19). The enzyme has been prepared in a highly purified form from potatoes by Boser (20).

Sidbury & Najjar (21) have determined the equilibrium constant for each of the two steps of the phosphoglucomutase reaction.

$$\text{glucose-1-phosphate} + \text{phosphate-enzyme} \rightleftharpoons \text{glucose-1,6-diphosphate} + \text{enzyme} \qquad 4.$$

$$\text{glucose-1,6-diphosphate} + \text{enzyme} \rightleftharpoons \text{glucose-6-phosphate} + \text{phosphate-enzyme} \qquad 5.$$

The value for the over-all K of the reaction is 17.2; the K of equation 4 and equation 5 is 4.58 and 3.76, respectively. The $\Delta F°$ values are, respectively, -0.91 kcal. for step 1 and -0.80 kcal. for step 2. The $\Delta F°$ for the hydrolysis of the enzyme-phosphate bond was calculated to be -3.9 kcal.

Phosphohexose isomerase.—Using *Phaseolus radiatus* as the source material, Ramasarma & Giri (22) have described the first purification of this enzyme isolated from a higher plant. The enzyme is rapidly inactivated when heated above 45°C. An unusual property of the enzyme is its pH optimum which is 7.8 with glucose-6-phosphate as the substrate and 9.0 with fructose-6-phosphate as the substrate. Using eviscerated animals as their test system, Wick *et al.* (23) have implicated phosphohexose isomerase as the site of 2-deoxyglucose inhibition. Bruns & Okada (24) have calculated the $\Delta F°$ for the phosphohexose isomerase reaction to be -0.084 kcal. from the equilibrium constant.

Fructose-1,6-diphosphatase.—Racker & Schroeder (25) have reported that

crude extracts of spinach leaves contain two fructose-1,6-diphosphatases. On purification, one is found to be located in the cytoplasm while the other is concentrated in the chloroplast. The cytoplasm enzyme has a pH optimum of 8.5; it is Mg^{++} dependent and is stimulated by ethylenediaminetetraacetate. These properties are similar to those of the purifiedliver enzyme [Mokrasch & McGilvery (26)]. Neither Mg^{++} nor ethylenediaminetetraacetate is required by the chloroplast enzyme of which the pH optimum is 6.9. Since fructose-1,6-diphosphate which is cleaved to fructose-6-phosphate and inorganic phosphate, appears to be its only substrate, the cytoplasm enzyme can serve as a useful analytic tool. However, the chloroplast enzyme will also cleave sedoheptulose-1,7-diphosphate at approximately twice the rate of fructose-1,6-diphosphate. This report strongly suggests that enzymes catalyzing similar reactions in the same cell may have different physical properties depending on their location. It would appear that cytoplasm enzymes concerned with the EMP pathway in the cytoplasm prefer a pH near eight while those catalyzing similar reactions in the chloroplast show a pH optimum closer to neutrality. Thus, Walker & Hill (27) have noted that the pH-activity curve for photosynthetic phosphorylation is pH 7 with a lower peak at pH 7.7, while Gibbs & Cynkin (28) have found that CO_2 assimilation by isolated spinach chloroplasts is highest at pH 7.4 with a rapid drop in activity as the pH increases. A reinvestigation of this problem might prove profitable.

Fructose-1,6-diphosphate aldolase.—This enzyme has been purified from *Aspergillus niger* until electrophoretically homogeneous by Jagannathan *et al.* (29). It resembles yeast aldolase rather than the aldolase of higher plants in that metal ions are required for activity. Inhibition by metal-binding agents was reversed by bivalent Zn, Fe, Mn, or Co. The enzyme has been detected in the green alga, *Hydrodictyon reticulatum*, by Richter (30).

D-*Glyceraldehyde-3-phosphate dehydrogenase.*—This enzyme continues to show its aberrance. Fuller & Gibbs (31) have followed up the speculation that photosynthetic tissues possess a TPN- as well as a DPN-linked dehydrogenase, as opposed to nonchlorophyll tissues which posses only the conventional DPN enzyme. As test photosynthetic systems they used *Chlorella*, *Chlamydomonas*, *Euglena*, *Rhodospirillum*, spinach chloroplasts, and *Romeria;* albino barley mutant, pea roots, *Tetrahymena*, streptomycin-bleached *Euglena*, dark-grown *Euglena*, and glucose-grown *Escherichia coli* were employed as test nonchlorophyll-containing systems. Only the albino barley mutant did not adhere to the general rule since it contained both dehydrogenases. Both dehydrogenases could be demonstrated in *Ulva lactuca* (19) while Trudinger (32) could find only the DPN enzyme in the strict chemoautotroph *Thiobacillus denitrificans.*

The significance of the three enzymes catalyzing the oxidation of the same substrate *in vivo* remains to be demonstrated. If 3-phosphoglyceric acid is the major carboxyl-containing compound reduced to the aldehyde

level during photosynthesis, it is difficult to implicate in this scheme, on thermodynamic grounds, the "new glyceraldehyde-3-phosphate oxidizing enzyme [Rosenberg & Arnon (33)]" since Rosenberg & Arnon (33) have claimed that it is irreversible. Their paper, however, does not show attempts to demonstrate reversibility using 1,3-diphosphoglyceric acid and their 84-fold purified enzyme.

Enolase.—The purification of *Pisum sativum* enolase by Miller (34) is the first report on the purification of this enzyme from a higher plant. A seven-fold purification over the crude extract of an acetone powder was recorded. The enzyme was also detected in the leaves of *Pisum*, *Chenopodium*, and *Nicotiana*, as well as the seeds of *Gossypium* and *Avena*. Its properties are similar to yeast enolase since it requires Mg^{++} for activity and is inhibited by a combination of Mg^{++}, F^- and P_i. The calculated K for enolase inhibition, using the method of Warburg and Christian gave 7×10^{-12} M^4 for pea seed enolase as compared to 3×10^{-12} M^4 for the yeast enzyme. Wold & Ballou (35, 36) have determined the equilibrium constant of the enolase reaction using, for the first time, the pure natural isomers of both substrates. An equilibrium constant of 6.3 was found for the reaction, 2-phosphoglyceric acid$\rightleftharpoons$phosphoenolpyruvic acid, which leads to a ΔF of -1.09 kcal.

Pyruvic acid kinase.—Thus far, there has been only indirect evidence that this enzyme exists in higher plants. Miller & Evans (37) have made the first report on some of the properties of the enzyme in crude preparations of plant material. Enzyme activity was assayed by the determination of pyruvic acid formed from phosphoenolpyruvic acid in the presence of ADP. The enzyme was shown to be distributed in leaves, embryos, seeds, and petioles. Detailed studies were reported only with the pea seed enzyme. For maximum activity, a divalent ion (Mg or Mn) and a monovalent ion (K, Rb or NH_4) is required. Ca^{++} is a relatively strong inhibitor of pyruvic acid kinase by competing competively for an active site with K^+ and Mg^{++}.

Lactic acid dehydrogenase.—While lactic acid has been known to exist in higher plants since the turn of the century, little information has been available on its formation. A DPN-linked enzyme has now been demonstrated in pea epicotyls (38), rice embryos (39), a particulate fraction from *Equisetum limosum* (40), and *Chlorella* (41). In the case of the *Chlorella* enzyme, the lactic acid was isolated as the zinc salt and shown to be dextrorotatory.

Routes Closely Related to the Embden-Meyerhof-Parnas Pathway

D-*Glyceric acid.*—This acid, presumably arising from phosphoglyceric acid by phosphatase activity, has been reported by Palmer (42) to be present in mature green leaves of *Nicotiana tabacum* to the extent of 0.5 to 1.5 per cent of the dry weight. This compound may also arise from D-glyceraldehyde catalyzed by a DPN-linked glyceraldehyde dehydrogenase, which was

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discovered by Lamprecht & Heinz (43) in rat liver. A D-glyceric acid kinase has been demonstrated in spinach leaves by Holzer & Holldorf (44) which would permit the free acid to enter the EMP pathway.

D-*Glyceric acid-3-phosphate.*—A pathway between glyceric acid-3-phosphate and phosphoserine via 3-phosphohydroxypyruvic acid has been demonstrated by Hanford & Davies (45) in crude extracts of eight- to ten-day-old pea epicotyls. These English workers have demonstrated in the extracts a dehydrogenase acting on 3-phosphoglycerate and a transaminase aminating 3-phosphohydroxypyruvic acid. They catalyze the following reactions:

$$\text{3-phosphoglyceric acid} + \text{DPN}^+ \rightarrow \text{3-phosphohydroxypyruvate} + \text{DPNH} + \text{H}^+ \quad 6.$$

$$\text{3-phosphohydroxypyruvate} + \text{L-glutamate} \rightarrow \text{3-phosphoserine} + \alpha\text{-ketoglutarate} \quad 7.$$

The dephosphorylation of 3-phosphoserine yields serine [Ichihara & Greenberg (46)]. These interesting findings strongly suggest that serine, which arises rapidly during photosynthesis from $C^{14}O_2$ with a tracer distribution similar to 3-phosphoglyceric acid, arises via 3-phosphohydroxypyruvic acid.

Phosphoenolypyruvic acid.—A significant development in plant biochemistry has been the discovery of a new connection between the EMP pathway and the citric acid cycle. This involves the carboxylation of phosphoenolpyruvic acid to form oxalacetic acid. Two different enzymes catalyze this reaction: (*a*) phosphoenolpyruvic acid carboxykinase [first described by Bandurski & Greiner (47, 48) in spinach] which is highly exergonic (equation 8) and (*b*) phosphoenolpyruvic acid carboxylase [first demonstrated by Utter & Kurahashi (49) in chicken liver] which catalyzes the reaction of equation 9 with a small loss in free energy, since energy is conserved in a pyrophosphate bond.

$$\text{phosphoenolpyruvic acid} + CO_2 \rightarrow \text{oxalacetate} + P_i \quad 8.$$

$$\text{phosphoenolpyruvic acid} + CO_2 + \text{GDP (or inosinediphosphate)} \rightarrow \text{oxalacetate} + \text{GTP (or inosinetriphosphate)} \quad 9.$$

A divergence from the animal system has been noted by Mazelis & Vennesland (50) who found that the phosphoenolpyruvic acid carboxykinase of higher plants uses ADP rather than GDP. A similar difference between the animal and plant has been demonstrated for the substrate-level phosphorylation accompanying α-ketoglutaric acid oxidation (1). In this same work, a wide distribution of the two enzymes in plant tissues was demonstrated. Phosphoenolpyruvic acid carboxylase was found to be concentrated in the particulate fraction (chloroplasts and mitochondria) of the spinach leaf. Anomalous results were obtained with spinach particulates when phosphoenolpyruvic acid carboxykinase was assayed. While investigating the ability of water extracts of spinach leaf chloroplasts to catalyze dark carboxylation reactions, Rosenberg *et al.* (51) found that the addition of phosphoenolpyruvate and $C^{14}O_2$ to the colorless extract caused the accumu-

lation of both isotopically-labeled oxalacetic acid and aspartic acid, confirming the apparent presence of a phosphoenolpyruvate carboxylation reaction in the particulate fraction. The presence of the phosphoenolpyruvate-carboxylating enzymes in the chloroplast may be the key to the suggestion made by Calvin & Massini (52) to explain their observation that light causes an inhibition of respiration of photosynthetic intermediates. They attributed this to a maintenance of α-lipoic acid in the reduced state, thereby causing a cessation of pyruvate oxidation. Using pyruvic acid-2-C^{14} and *Scenedesmus*, Milhaud *et al.* (53), working in the same laboratory, modified this suggestion and concluded that two sites of pyruvic acid oxidation existed in the green cell. One, located in the chloroplast, was blocked by light, while the extrachloroplastic site could proceed both in the light as well as in the dark. This reviewer would like to propose another explanation for these interesting observations. The strong light inhibition of pyruvic acid oxidation observed by the California group may be due to a lack of phosphoenolpyruvate, since exogenous CO_2 is being reduced to carbohydrate at a rate 30 to 40 times more rapid than the dissimilation of the carbohydrate. When the "reducing power" is limiting or completely eliminated, the photosynthetic carbon cycle rate is accordingly diminished; phosphoenolpyruvate is then available for carboxylation and tracer enters a cycle whose rate is infinitely high compared to carbohydrate formation. Another point in favor of this interpretation is the observation of Arnon (54) that the chloroplast does not contain enzymes of the citric acid cycle. Therefore, it would appear that the phosphoenolpyruvate-carboxylating enzymes may be important not only to crassulacean metabolism (see below) but also to the photosynthetic cell in providing the bridge between chloroplast and cytoplasm metabolism.

Results from various laboratories [Walker (55), Mazelis & Vennesland (50)] suggest that the two carboxylating enzymes coupled to malic dehydrogenase may act as the bridge between carbohydrate and malic acid in the well-known acid metabolism of the succulents. In addition, Saltman *et al.* (56) have noted that intact leaves of *Bryophyllum calycinum* can incorporate CO_2 into oxalacetate. Although this type of evidence is suggestive, an alternative scheme involving the "malic enzyme" could yield similar results in the succulent plant.

Another reaction involving phosphoenolpyruvate is its condensation with D-erythrose-4-phosphate to yield 2-keto-3-deoxy-7-phosphoglucoheptonic acid (KDPH) [Srinivasan & Sprinson (57)]. The enzyme responsible for the first step in the conversion of these two compounds to shikimic acid [Srinivasan *et al.* (58)] is phosphoenolpyruvate-erythrose-4-phosphate aldolase. The enzyme isolated from *Escherichia coli* has a K_m for phosphoenolpyruvate of 2.0×10^{-3} M, while that for erythrose-4-phosphate is 2.6×10^{-3} M. This aldolase, unlike fructose-1,6-diphosphate aldolase, is specific for these two substrates. D-Erythrose, glucose-6-phosphate, ribose-5-phosphate, and glyceraldehyde-3-phosphate cannot substitute for erythrose-4-phosphate, while pyruvic acid cannot replace phosphoenolpyruvate. No cofactor re-

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quirements could be observed. Rothschild (59), working with this same group has published a preliminary note on the quantitative conversion of KDPH to 5-dehydroquinic acid by *E. coli* extract. The 5-dehydroquinic acid is apparently an intermediate between KDPH and shikimic acid. In crude extracts, 10^{-2} *M* versene inhibits the synthesis of 5-dehydroquinic acid from KDPH with the concomitant release of one mole of P_i and a new intermediate which is still an α-keto acid. The distribution of tracer in protocatechuic acid produced by a nonaromatic strain of *Neurospora crassa* grown on glucose-1-C^{14} and glucose-6-C^{14} led Tatum & Gross (60) to the conclusion that aromatization in *Neurospora* and *E. coli* involves essentially similar pathways. These observations are of considerable interest since it provides the first proof of a direct connection between products of carbohydrate dissimilation and the simpler aromatic compounds.

The Pentose Phosphate Pathway

Until 1945, it was generally felt that the initial steps in carbohydrate dissimilation in cells were catalyzed by the EMP pathway. The first unequivocal proof that the glycolytic mechanism could depart from the usual EMP pattern of reactions was derived from observations with the heterofermentative lactic acid bacterium, *Leuconostoc mesenteroides* [De Moss *et al.* (61), Gunsalus & Gibbs (6)]. That this departure from the EMP pathway was not limited to the anaerobic bacteria was soon clearly evident. Enzymatic as well as isotopic evidence has come from many sources, strongly suggesting that most tissues dissimilate carbohydrate by multiple pathways. Embryonic tissue of plants may be an exception. In addition to the EMP pathway, the pentose phosphate pathway appears to have the most potential significance.

The pentose phosphate pathway is considered in this review to include both the enzymes catalyzing the formation of CO_2 and ribulose-5-phosphate from glucose-6-phosphate, which was formerly termed the hexose monophosphate pathway (HMP), and also the group of enzymes catalyzing the rearrangements of pentose phosphate leading to a resynthesis of glucose-6-phosphate.

Quantitative importance of the pentose phosphate pathway.—Many experiments have been designed to elucidate the pathways of glucose utilization and also to determine the degree to which one pathway, of the several present, functions in the intact organism. The isotopic methods fall into two types, one measuring the total activity of metabolically-produced CO_2 and the second depending on the isolation and degradation of a breakdown product of glucose. The most commonly used technique [Bloom & co-workers (62)] takes advantage of the fact that glucose molecules entering the HMP pathway give rise to pentose phosphate and CO_2 with the CO_2 originating from carbon atom 1 of the glucose; glucose molecules entering the EMP pathway and citric acid cycle give rise to CO_2 at an equal rate from carbon atoms 1 and 6. As Wood (63) points out, this method weighs the calcula-

tions heavily in favor of the HMP pathway since the glucose traverses only three intermediates (6-phosphogluconolactone, 6-phosphogluconate and ribulose-5-phosphate) before $C^{14}O_2$ is released by this pathway, whereas, some twenty intermediate compounds intervene before $C^{14}O_2$ is released by the EMP pathway and citric acid cycle. Opinions of this type have been expressed in numerous papers and the preliminary nature of the conclusions is realized by most investigators.

The methods depending on the isolation and degradation of two or three carbon fragments yielded by labeled glucose was proposed by Blumenthal *et al.* (64). To bring about the formation of these intermediates, it is either necessary to trap intermediary products in an added pool [Lewis *et al.* (65)] or to use a selective inhibitor such as arsenite to cause pyruvic acid to accumulate [Dawes & Holms (66)]. Figure 1 illustrates the EMP and HMP pathways of glucose catabolism and the distribution of the carbon atoms of glucose in pyruvic acid and ethanol formed by these pathways. The Entner-Doudoroff system has been omitted since present evidence indicates that this pathway is limited to a few microorganisms.

This approach like the previous one has certain disadvantages. Recycling of the intermediates and the use of an inhibitor are two of many. This method, which has been applied principally to microorganisms, has been used to investigate corn root metabolism (5). In the reviewer's laboratory the method has given negative results when it was applied to leaves of higher plants (unpublished observations).

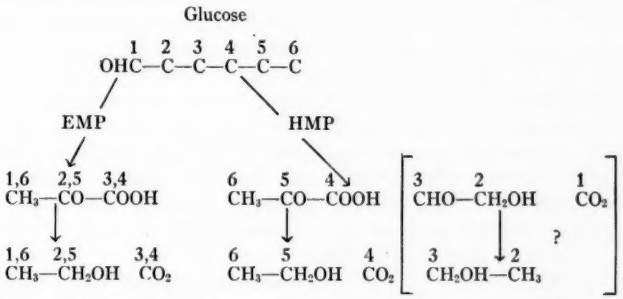

FIG. 1. Distribution of the carbon atoms of glucose in pyruvic acid, ethanol and CO_2 formed by the Embden-Meyerhof-Parnas and hexose monophosphate pathways of glucose dissimilation. The numbering of the carbon atoms corresponds to those of the original glucose. Distribution of carbon positions 2 and 3 by recycling through transaldolase and transketolase is not assessed.

Daly *et al.* (67) have used the Bloom, Stetten, and Stetten procedure to assess the characteristically large increases in respiratory rates of higher plants infected by obligate plant parasites. Using healthy and rust-infected hypocotyls of safflowers as test tissue, they concluded that nearly all the in-

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creased respiratory activity arising from infection is mediated by the HMP pathway. A similar finding was reported for wheat and barley leaves infected with *Puccinia graminis* and *Erysiphe graminis* [Shaw & Samborski (68)]. With glucose-1-C^{14} and glucose-6-C^{14} as indicators of the pathway of glucose catabolism (C_6/C_1 ratio), the effect of 10^{-3} *M* 2,4-D on the carbohydrate metabolism of pea, corn, and oat seedling, was studied by Humphreys & Dugger (69). In all seedlings, 2,4-D caused an increase in the amount of glucose respired through the HMP system as indicated by a decrease in the C_6/C_1 ratio. This decrease could result from a lower yield of $C^{14}O_2$ from glucose-6-C^{14} (the yield from glucose-1-C^{14} remaining unchanged), from a higher yield from $C^{14}O_2$ from glucose-1-C^{14} (the yield from glucose-6-C^{14} remaining unchanged), or from a combination of a higher yield with glucose-1-C^{14} and a lower yield from glucose-6-C^{14}. Their data illustrate all three cases depending on the way in which the seedlings were treated. For instance, root tips obtained from intact seedlings behaved differently than those obtained from seedlings which, prior to 2,4-D treatment, had their cotyledons removed. The C_6/C_1 values obtained with untreated corn root tips varied over a wide range (0.47 to 1.02). This variation appeared to be correlated with the moisture conditions under which the seedlings were grown. If the corn was germinated with a limiting amount of moisture, the ratios were 0.5 to 0.6; the ratios approached 1.0 when the seedlings were kept wet. The latter data are in agreement with those of Beevers & Gibbs (70).

Indoleacetic acid does not appear to influence glucose catabolism in the same manner as 2,4-D. Shaw *et al.* (71) confirmed the observation of Gibbs & Beevers (72) that a low concentration of indoleacetic acid (1.7–3.3 mg./l.) stimulated the growth of plant tissue with a concomitant increase in oxygen consumption but had no appreciable effect on the C_6/C_1 ratio. It was found (71), however, that somewhat higher concentrations of indoleacetic acid (5.5–16.6 mg./l.) lowered the C_6/C_1 ratio. When Humphreys & Dugger (69) treated corn and pea root tips with an extremely high concentration (164 mg./l. $= 10^{-3}$ *M*) of indoleacetic acid, the C_6/C_1 obtained was markedly lowered. Shaw *et al.* (71) concluded, as Gibbs & Beevers (72) had done previously, that the HMP pathway plays a more prominent role in glucose dissimilation as the higher plant matures.

Tager (73) has observed a shift to the EMP pathway from the pentose phosphate pathway during the climacteric rise in the ripening banana. The metabolic pattern changes with the appearance of fructose-1,6-diphosphate aldolase, pyruvic acid carboxylase, and a sensitivity of the respiration of the pulp to fluoride.

It is difficult to assess the biochemical significance of such effects on the C_6/C_1 ratio. It is not clear that there is any direct relation between these effects and the mechanism of auxin action. At the present, there is no available evidence that indoleacetic acid has any effect on enzymes of either pathway. However, it is attractive to speculate that indoleacetic acid may regulate the pathway of glucose dissimilation.

DeMoss *et al.* (61) have shown that acetate, acetaldehyde, acetone, and dihydroxyacetone stimulate the rate of glucose fermentation by *Leuconostoc mesenteroides*, a coccus which ferments exclusively by a HMP pathway. Bovine corneal epithelium has been reported by Kinoshita (74) to shunt more glucose-1-C^{14} from the EMP pathway to the HMP pathway, as evidenced by an eightfold increase in $C^{14}O_2$ production upon the addition of a TPNH acceptor, in this case, pyruvate. Since both the microorganism and the corneal epithelium lack pyridine nucleotide transhydrogenase, it would appear that an oxidation of TPNH is coupled to the reduction of pyruvate. Since TPN-linked alcohol dehydrogenase has been reported in *Leuconostoc mesenteroides* (75), this would suggest that lactic acid dehydrogenase is capable of functioning with TPN in the bovine tissue. The observation of Barron & Harrop (76), made nearly 30 years ago, that the respiration of nonnucleated erythrocytes may be stimulated by the addition of methylene blue, has been explained by Brin & Yonemoto (77). These workers showed that the rate of oxygen consumption and the rate of $C^{14}O_2$ production from glucose-1-C^{14} and glucose-2-C^{14} varied directly with the concentration of the dye. These data, together with the lack of $C^{14}O_2$ from glucose-6-C^{14}, indicated that methylene blue activated at least a portion of the pentose phosphate pathway. A report by Langdon (78) indicates that fatty acid synthesis in the rat liver may be coupled to the pentose phosphate cycle through TPN-TPNH. He has shown that the incorporation of acetate-1-C^{14} into fatty acids by a soluble fraction of rat liver is stimulated by TPNH. In this process, TPNH serves as an electron donor for the reduction of α,β-unsaturated acyl CoA derivatives to their saturated counterparts. This type of approach has not been applied to tissues of higher plants.

Evidence of operating the complete pentose phosphate pathway cycle in vivo.—The proof that the pentose phosphate pathway or cycle represents a mechanism for the complete oxidation of glucose appears to be available. This metabolic pattern would involve the oxidation of glucose-6-phosphate to 6-phosphogluconate and pentose phosphate and the resynthesis of fructose-6-phosphate by the action of transaldolase and transketolase [Horecker (79) and Racker (80)]. During one turn of this cycle, one CO_2 is released and two moles of pyridine nucleotide are reduced, the latter in turn being oxidized by a mole of O_2 resulting in a R.Q. of 1. By further cycling, carbon atoms 2 and 3 of the fructose-6-phosphate yield CO_2 successively. Thus, fructose-6-phosphate or glucose-6-phosphate, after isomerization to fructose-6-phosphate, could be completely oxidized by repetition of this reaction series, without other steps of the EMP pathway or the citric acid cycle. The net result is:

$$3 \text{ glucose} \rightarrow 3\ CO_2 + 2.5 \text{ glucose} \qquad 10.$$

Kitos *et al.* (81) have determined the rate of $C^{14}O_2$ production from uniformly and specifically labeled glucose by *Acetobacter suboxydans*. They concluded that rapid and successive elimination of carbon atoms 1 and 2, to-

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gether with the slow release of carbon atom 6, was proof that the pentose phosphate cycle was a mechanism of prime importance to this microorganism for the complete oxidation of glucose.

In another investigation, Muntz & Murphy (82) have developed an elegant method for the study of the metabolic reactions that occur in liver *in vivo*. The method made use of brief, intraportal injection periods of labeled glucose followed by a rapid removal of the rat liver. The distribution of tracer in the liver lactic acid was used to estimate the extent to which the various pathways participate in the metabolism of glucose. Table 1 contains representative values.

TABLE 1

REDISTRIBUTION OF C^{14} AS OBSERVED IN LIVER LACTATE AFTER INJECTION OF LABELED GLUCOSE

Type of glucose injected	Percentage of radioactivity present in each position		
	Carboxyl carbon	α-carbon	β-carbon
Glucose-3,4-C^{14}	94.6	5.1	0
	90.6	6.6	2.8
Glucose-2-C^{14}	3.7	91.4	4.9
	2.3	96.4	1.3
Glucose-2,6-C^{14}	2.5	38.3	59.2
	6.0	39.7	54.3

As shown in Table 1, some redistribution does occur. From glucose-3,4-C^{14}, carbon 3 moved predominantly to the 2 position and thereby caused about five to six per cent labeling in the α-carbon of the lactate. From glucose-2-C^{14} the tracer moved to the carboxyl and β-carbons of lactic acid in accordance with the data of Horecker *et al.* (83). However, as Muntz & Murphy point out, the conversion of carbon 2 to carbon 1 via the HMP pathway did not occur to a major extent during the short experimental period (90 sec.) since only one to five per cent of the activity was in the β-carbon of lactate from glucose-2-C^{14}. With glucose-2,6-C^{14} (a mixture of glucose-2-C^{14} and -6-C^{14}), it was felt that an estimate of participation of the two pathways was possible since operation of the EMP pathway would give lactate equally labeled in the α and β positions, while the HMP oxidation pathway would give the β-labeled lactate. The α-carbon is a measure of the contribution of the EMP pathway in contrast to the β-carbon which reflects the operation of both pathways. Therefore, the difference in activity divided by the activity in the β-carbon is the fraction of lactic acid contributed by the HMP pathway. It was estimated that 29 to 38 per cent was catabolized by a pathway other than the EMP pathway. Because of the

assumptions made in this type of experiment, the tentative nature of these values is stressed by the authors.

Black *et al.* (84) have performed a similar type of study by injecting lactating cows intravenously with glucose-1-C^{14} and glucose-6-C^{14} and then comparing the amount of tracer in the respired CO_2 and the distribution of tracer in the alanine and serine from casein and in the glycerol from the milk fat. The quantitative significance of the pentose phosphate cycle estimated from alanine was 40 per cent in contrast to a value of 50 to 65 per cent from serine or glycerol. The recent experiences of Wood & co-workers with asymmetrical labeling in the lactose of lactating cows could have an influence on these values.

Higher plants have not been treated by this technique, which could give an insight into the effect of light on the pathways of carbohydrate dissimilation. The reviewer is unaware of data indicating the presence of C^{14} in 6-phosphogluconate during short-time photosynthesis experiments with $C^{14}O_2$ using higher plants or algae.

Role of ribulose-5-phosphate.—The most significant observation in this area of carbohydrate metabolism since the review of Axelrod & Beevers (1) is a re-evaluation of the importance of a ribulose-5-phosphate as the key compound undergoing cleavage catalyzed by transketolase. In 1955, Srere *et al.* (85) found crystalline yeast transketolase to be inactive with ribulose-5-phosphate and indicated that the substrate for cleavage was D-xylulose-5-phosphate. Horecker *et al.* (86) quickly confirmed this report. Purification of their spinach transketolase revealed the presence of a phosphoketopentoisomerase which converted ribulose-5-phosphate to xylulose-5-phosphate (Hurwitz & Horecker (87)]. This phosphoketopentoisomerase had been detected previously using spleen (88) and muscle preparations (89). It is now clear that yeast, liver, and spinach transketolase require xylulose-5-phosphate for activity in addition to an "active glycolaldehyde" acceptor. Other substrates which give rise to "active glycoaldehyde" like L-erythrulose and fructose-6-phosphate have a configuration identical with that of xylulose and sedoheptulose in carbon atoms 1 and 4. L-Ribulose-5-phosphate, which differs only with respect to carbon atom 4, is inactive (see below). Figure 2 depicts the pentose phosphate scheme as it is now thought to function.

Racker & Schroeder (90) have shown that transketolase and transaldolase can form and utilize octulose-8-phosphate by the following reactions:

$$\text{ribose-5-phosphate} + \text{fructose-6-phosphate} \rightleftharpoons \text{glyceraldehyde-3-phosphate} + \text{octulose-8-phosphate} \qquad 11.$$

$$\text{octulose-8-phosphate} + \text{glyceraldehyde-3-phosphate} \rightleftharpoons \text{xylulose-5-phosphate} + \text{allose-6-phosphate} \qquad 12.$$

The presence of the postulated allose-6-phosphate was not established.

A new and interesting enzymatic reaction specific for D-xylulose-5-phos-

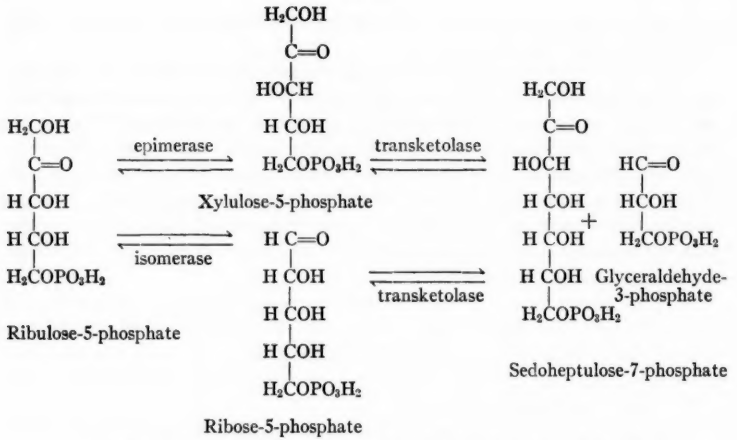
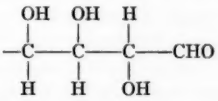


FIG. 2. The relation between D-ribulose-5-phosphate, D-xylulose-5-phosphate and D-ribose-5-phosphate.

phate has been demonstrated by Heath *et al.* (91). Extracts of *Lactobacillus plantarum* carry out a TPP-dependent phosphorylitic cleavage of xylulose-5-phosphate to form acetyl phosphate and triose phosphate. The enzyme, called phosphoketolase, occurs with a loss in free energy calculated to be 13 kcal. The value is consistent with the observed lack of reversibility of the over-all reaction. The enzyme does not react with fructose-6-phosphate. This contrast with the enzyme isolated from *Acetobacter xylinum* by Schramm & Racker (92) which catalyzes not only a phosphorylitic cleavage of xylulose-5-phosphate but also of fructose-6-phosphate, the latter resulting in the formation of acetyl phosphate and erythrose-4-phosphate. Neither group speculated on the mechanism whereby a high-energy phosphate bond is created during cleavage.

Pentose interconversions.—A considerable number of complete reports have appeared concerning the metabolism of L-arabinose. In each investigation, bacteria adapted to L-arabinose have been employed.

Neish & Simpson (93) and Altermatt *et al.* (94) found that *Aerobacter aerogenes* ferments L-arabinose-1-C^{14}, D-arabinose-1-C^{14}, D-ribose-1-C^{14}, and D-xylose-1-C^{14} to identical products with a similar labeling pattern. This suggests that the L-form is metabolized via an intermediate common to the D-pentoses. Mitsuhashi & Lampen (95) observed that extracts of *Lactobacillus pentosus* isomerize L-arabinose to L-ribulose, followed by a phosphorylation of the ketopentose. An isomerase which catalyzes the reversible reaction—L-arabinose⇌L-ribulose—has been purified from extracts of *Lactobacillus plantarum* (96) and *A. aerogenes* (97). The isomerase appears to be specific for the group,



13.

and at equilibrium about 90 per cent of the total pentose is present as L-arabinose. A kinase capable of phosphorylating ribulose-5-phosphate has been purified from *A. aerogenes* (98) and *L. plantarum* (99). Although specific for the pentose, ribulose, it is active with both D and L isomers and therefore has been referred to as ribulokinase. Extracts of the two organisms also catalyze the conversion of L-ribulose-5-phosphate to D-xylulose-5-phosphate (100, 101). The enzyme has been called L-ribulose-5-phosphate-4-epimerase because it catalyzes a change in configuration at the 4 position. Since the reaction is similar to that catalyzed by UDPG-4-epimerase, the name L-ribulose-5-phosphate-4-epimerase is preferred to stereoisomerase (100). The 4-epimerase is inactivated by *Neurospora* diphosphopyridine nucleotidase but the inactivated enzyme cannot be reactivated by DPN or by boiled bacterial extracts. These reactions are summarized in Figure 3. The path of D-arabinose breakdown is still unknown.

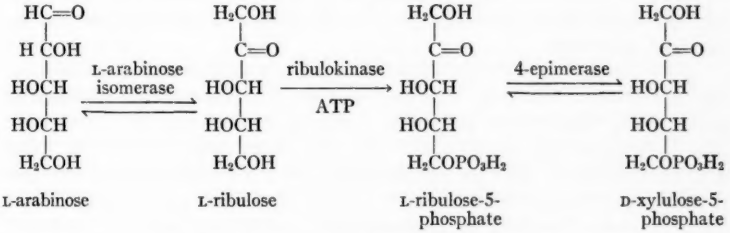


FIG. 3. The pathway of L-arabinose metabolism.

A D-xylose isomerase which catalyzes the reversible formation of D-xylulose from D-xylose has been reported by Stone & Hochster (102) and by Slein (103) in extracts of xylose-grown cells of *Pseudomonas hydrophila* and *Pasteurella pestis*. Extracts of *P. hydrophila* also contain a kinase which catalyzes the phosphorylation of D-xylulose to yield a mixture of phosphate esters (104). The enzyme, D-xylulokinase, has been purified by Stumpf & Horecker (105) from extracts of xylose-grown *Lactobacillus plantarum* and by Hickman & Ashwell (106) from mammalian liver. The enzyme in both investigations was found specific for D-xylulose.

The important studies of Hollman & Touster (107) revealed the presence of an enzyme system in guinea pig liver mitochondria capable of reversibly reducing both L- and D-xylulose to a common intermediate, xylitol, thereby providing a mechanism for the interconversion of the stereoisomers of this pentose.

$$\text{xylitol} + \text{TPN}^+ \rightleftharpoons \text{L-xylulose} + \text{TPNH} + \text{H}^+ \qquad 14.$$

The TPN-linked enzyme which is specific for L-xylulose and xylitol has an apparent equilibrium constant of 8.58×10^{-11} *M*. The authors suggested that the enzyme be named "L-xylulose (xylitol) reductase."

In contrast to the TPN enzyme, the DPN-requiring dehydrogenase, which has an equilibrium constant of 1.55×10^{-11} *M*,

$$\text{xylitol} + \text{DPN}^+ \rightleftharpoons \text{D-xylulose} + \text{DPNH} + \text{H}^+ \qquad 15.$$

will catalyze the interconversions of several substrates, namely, L-threitol and L-erythrulose, ribitol and D-ribulose, D-sorbitol and D-fructose, L-iditol and L-sorbose, and D-glycero-D-glucoheptitol and a heptulose. It is clear that the directional flow will depend on the relative concentrations of TPNH/TPN and DPNH/DPN. Glock & McLean (108) have determined the levels of reduced and oxidized coenzymes in animal tissues. In all tissues tested, TPN was present in the reduced state while DPN occurred predominantly in its oxidized form. An investigation of this type with higher plants would be most useful since, as discussed by DeDuve & Hers (109), steady-state concentrations of the coenzyme could be a means of regulating metabolic flow. In a preliminary note, Chiang *et al.* (110) have demonstrated that extracts of *Pencillium chrysogenum* contain a TPNH-specific enzyme catalyzing the reduction of D-xylose to xylitol. Xylose isomerase could not be detected in the extracts.

Deoxyribose.—In contrast to ribose, relatively little is known concerning the metabolism of 2-deoxy-D-ribose. Racker (111) was the first to describe the enzyme, deoxyribose-5-phosphate aldolase, in extracts of *Escherichia coli*, *Corynebacterium diphtheriae*, and mouse liver, which reversibly splits deoxyribose-5-phosphate to acetaldehyde and glyceraldehyde-3-phosphate. A similar enzyme has been demonstrated by Howells & Lindstrom (112) in *Bacillus cereus*. Hoffman & Lampen (113) concluded from stoichiometry data that a similar type of cleavage was involved in the fermentation of deoxyribose nucleosides by *E. coli*. The formation of stoichiometric quantities of acetaldehyde and lactic acid during the fermentation of deoxyribose by *Lactobacillus planatarum* in the presence of bisulfite has been given as evidence of a similar cleavage in this microorganism [Domagk & Horecker (114)]. In addition, extracts of the organism were shown to contain deoxyribose-5-phosphate aldolase. A preliminary note by Shreeve & Grossman (115) indicates that deoxyribose-5-phosphate aldolase plays an important role in the incorporation of glycine-1-C^{14} into the deoxyribose of DNA in rat liver. Rose & Schweigert (116) and Roll *et al.* (117) have reported tracer experiments suggesting that the intact rat can transform pyrimidine ribosides to pyrimidine deoxyribosides without rupture of glycosidic linkages.

Bernstein & Sweet (118) have obtained results suggesting that deoxyribose phosphate aldolase does not play a major role in the synthesis of the deoxyribose of *Escherichia coli* grown on glucose or lactic acid. The patterns of C^{14} found in the deoxyribose of thymidine and adenosine indicate that the loss of carbon atom 1 of hexose is the major route of pentose formation. A

comparison of the distributions of tracer in the deoxypentose with those found in ribose suggest that ribose is reduced directly to the deoxypentose or that both have a common precursor. Typical of such experiments is the conclusion that "neither of these explanations, however, accounts for certain aspects of the data." It is interesting to note that deoxyribose phosphate aldolase has been demonstrated in this strain of *E. coli*. In a similar investigation, Bagatell *et al.* (119) have obtained identical labeling of ribose and deoxyribose derived from *E. coli* R2 grown on acetic acid-1-C^{14} as the sole carbon source. Recent evidence obtained with broken-cell preparations are suggestive of a direct interconversion of the two types of pentose. Thus, Grossman & Hawkins (120) have demonstrated that a soluble extract of *Salmonella typhimurium* LT2 contains enzyme systems which catalyze the conversion of uridine and cytidine to deoxyuridine and deoxycytidine. These same extracts have been shown by Grossman (121) to catalyze the conversion of cytidine-5′-phosphate to deoxycytidine-5′-phosphate, purine ribosides to purine deoxyribosides, and purine ribotides to purine deoxyribotides. Reichard (122) has presented evidence for the existence of a similar reaction in the extracts of chicken embryos. When chicken embryo homogenates were incubated with 2-C^{14}-uridine-5′-phosphate, a radioactive spot corresponding to deoxyuridine-5′-phosphate was located on a paper chromatogram.

The decarboxylation of 2-keto-3-deoxy-6-phosphogluconate, the key intermediate of the Entner-Doudoroff pathway, could lead to deoxyribose phosphate [Lanning & Cohen (123)]. At present there is no evidence for the existence of such an enzyme. This mechanism would appear to be limited since the Entner-Doudoroff scheme has been detected in only a few bacteria.

There is no evidence in the literature that the biosynthesis of deoxyribose in higher plants has received attention.

Cyclic Pathways for Glucose Metabolism

Hexose-pentose cycle.—A second hexose-pentose cyclic pathway for the oxidation of glucose has been tentatively established solely in mammalian tissues. Some of the reactions have been demonstrated in higher plants. In contrast to the "classical" pentose phosphate pathway, this cycle is characterized by a decarboxylation involving C-6 of the hexose and by nonphosphorylated intermediates. The cyclic nature of these reactions is indicated in Figure 4. Tracer evidence for this cycle in the intact rat and guinea pig has been given by Dayton *et al.* (124). Utter (125) has suggested the name glucuronate xylulose pathway.

The oxidation of D-glucose to D-glucuronate is presumably catalyzed by a UDPG dehydrogenase (126), the mechanism of which will be discussed under glucuronic acid metabolism. Partially purified preparations from hog kidney have been shown by Bublitz *et al.* (127) to catalyze a TPN-specific reversible reduction of D-glucuronate to L-gulonate. The γ-lactones were reported in this same work to be attacked only after hydrolysis. This is in

contrast to others (128) who have demonstrated that the lactones are more readily metabolized. When L-gulonate and DPN were incubated with rat liver or hog kidney fractions, then DPNH, CO_2, and xylulose were formed. The reaction in these preparations is specific for DPN and for the L-stereoisomer of gulonate. These data are in agreement with those reported from other mammalian tissues (128, 129). An inspection of the reaction sequence has led to the suggestion that 3-keto-L-gulonic acid is the hypothetical intermediate in the formation of L-xylulose from L-gulonic acid. That part of this cycle may be present in higher plants is suggested by the study of

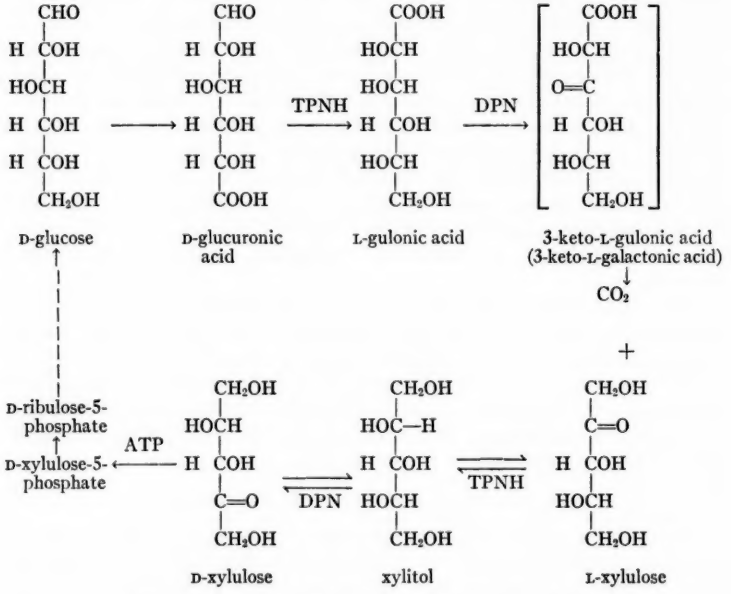


FIG. 4. Cyclic pathway for glucose oxidation involving glucuronate and xylulose as key intermediates.

Finkle & Loewus (130). Experiments in which D-glucuronolactone-1-C^{14} and -6-C^{14} were administered to detached, ripening strawberries suggest that these compounds are metabolized in the berry as 3-keto-L-gulonate and its decarboxylated product, L-xylulose. While glucuronolactone-1-C^{14} yielded labeled xylose and $C^{14}O_2$, the 6-labeled γ-lactone gave rise to unlabeled xylose and $C^{14}O_2$. A more complete report of this work will be awaited with interest.

The conversion of L-xylulose to hexose phosphate via D-xylulose-5-phosphate, the intermediate common to both this hexose-pentose pathway and the pentose phosphate pathway, has been discussed earlier.

Ascorbic acid.—In mammalian tissues, this new pathway is closely linked to L-galactonate and L-ascorbate through the hypothetical intermediate, 3-keto-L-gulonate (3-keto-L-galactonic acid). The reactions to be discussed in this section are outlined in Figure 5.

The mechanism of conversion of D-galactose to D-galacturonic acid is still unknown. An extract of rat liver acetone powder has been shown by

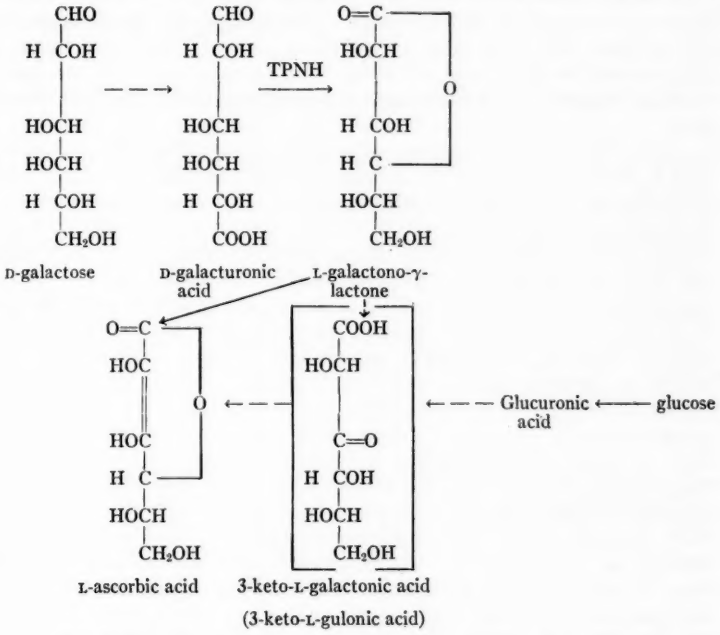


FIG. 5. Biosynthesis of L-ascorbic acid from D-glucose and D-galactose.

Ashwell (131) to be capable of metabolizing D-galacturonic acid in the presence of TPNH. The oxidation product was not characterized but by analogous reactions it is assumed to be L-galactono-γ-lactone. Since after mild acid hydrolysis the reaction product was L-xylulose, it was postulated that another intermediate compound, 3-keto-L-galactonic acid, was formed because decarboxylation of the lactone would yield a product of lower oxidation level than a ketopentose.

Mapson & Breslow (132) have purified from cauliflower mitochondria a highly specific dehydrogenase which converts L-galactono-γ-lactone to L-ascorbic acid. An enzyme specificity for the configuration around position 2 and 3 of the aldonic acid lactone is indicated since L-gulono-γ-lactone was not attacked. In addition, the enzyme is active only toward the L-isomer. These results contrast with the relatively low specificity of the enzyme

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demonstrated in cress seedlings (133) and pea mitochondria (134). Whereas this enzyme is inactive toward the free acid, mitochondrial preparations from rat liver have been reported to form ascorbic acid from the free acid as readily as from the lactone (135). A requirement for inorganic phosphate and the spectral changes accompanying the reduction of substrate, among other characteristics, suggested a flavoprotein similar to succinic dehydrogenase (136); however, its insensitivity to most metal-bindings agents with the exception of *o*-phenanthroline indicated a ferroflavoprotein. The detection of this enzyme, which apparently synthesizes L-ascorbic acid directly from L-galactono-γ-lactone thus excluding 3-keto-L-galactonic acid as intermediate, suggests that ascorbic acid synthesis may differ in various organisms.

This difference in the pathway of ascorbic acid synthesis between organisms is borne out strikingly in a comparison of the distribution of tracer in ascorbic acid derived from variously labeled substrates. In the conversion of glucose-1-C^{14} to ascorbic acid in the rat, the observation was made by Horowitz *et al.* (137) that, while the carbon chain remained intact, C^{14} appeared in ascorbic acid predominantly labeled in carbon atom 6. This apparent inversion of the carbon chain is presumably caused by the oxidation of carbon atom 6 of glucose to form glucuronic acid, with subsequent reduction of position 1 to yield L-gulonic acid. When a similar study was applied to germinating cress seedlings [Loewus & Jang (138)] and strawberries [Loewus & co-workers (139)], it was found that no inversion occurred. Ascorbic acid obtained from these plant materials given glucose-1-C^{14} contains most of its radioactivity in carbon atom 1. A series of interesting experiments with the detached, ripening strawberry in which glucose-2-C^{14}, galactose-1-C^{14}, arabinose-1-C^{14}, xylose-1-C^{14}, glucuronic acid-6-C^{14}, glucuronolactone-6-C^{14}, and bicarbonate-C^{14} were fed, and from which labeling patterns of the free sugars, ascorbic acid, and galacturonic acid were obtained [Loewus & co-workers (140, 141)] strongly suggests that 6-phosphogluconate and not glucuronic acid or galacturonic acid is the ultimate precursor of ascorbic acid. A recent preliminary note from this same group indicates that ripening berries may also contain the pathway involving inversion of the tracer (130).

Hexose-triose phosphate cycle.—A new "hexose cycle" has been proposed by Hochster & Katznelson (142) for cells which tend not to utilize the pentose phosphate cycle or EMP pathway but have an active 6-phosphogluconate cleaving system (Entner-Doudoroff) yielding triose phosphate and pyruvic acid which are then broken down for energy. The pentose phosphate cycle and the entire EMP pathway would have a limited role. This cycle, which is outlined in Figure 6, is probably limited in distribution.

Pathways initiated by direct oxidation of glucose.—The oxidation of hexoses prior to phosphorylation to yield the corresponding acid, previously thought to be present only in heterotrophic tissue, has now been reported in the photosynthetic marine red alga, *Iridophycus flaccidum* [Bean & Hassid (143)]. The algal enzyme appears similar to the D-glucose oxidase (notatin)

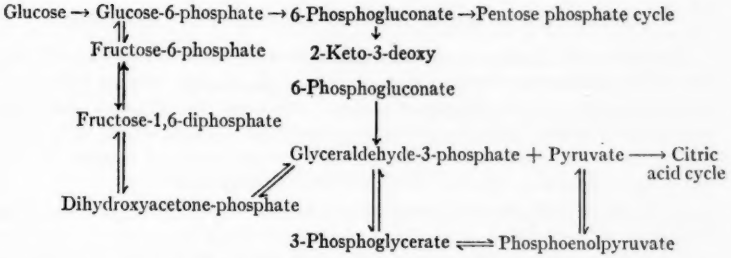


FIG. 6. Hexose-triose phosphogluconate cycle as a major metabolic pathway in microorganisms possessing an active Entner-Doudoroff cleaving system.

since H_2O_2 is generated. However, in its substrate specificity, it differs radically from all the other known oxidases. The fact that the *Iridophycus* enzyme is capable of oxidizing D-glucose and D-galactose but not D-mannose indicates that the enzyme is specific with regard to the C-2 configuration and is nonspecific with regard to that of C-4 in the hexose chain. Since this enzyme is also capable of oxidizing lactose, maltose, and cellobiose at the same rate as glucose, the hydroxyl of C-4 may be substituted by a D-glucose or D-galactose unit. The enzyme is not active toward D-xylose and L-arabinose. The primary C-6 hydroxyl appears to be indispensable since galacturonic acid is not oxidized. The significance of a direct oxidation of sugars in higher plants remains to be demonstrated. It may be of interest to summarize (Fig. 7) the recent developments in this field since this information will be of value to the future studies of plant biochemists. All of the enzymes furthering the metabolism of gluconate are of bacterial origin (144). There is a note to the fact that barley germ does not metabolize 2-keto-6-phosphogluconate [DeLey & Verhofstede (145)].

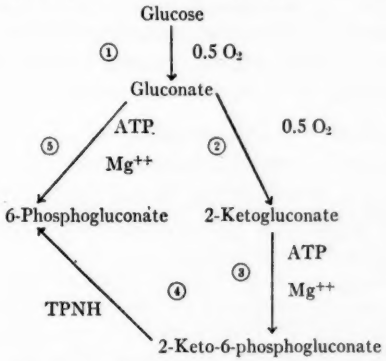


FIG. 7. Reactions involving gluconate. The individual reactions are catalyzed by the following enzymes: 1. glucose oxidase; 2. gluconate oxidase; 3. 2-keto-gluconokinase; 4. 2-keto-6-phosphogluconate reductase; 5. gluconokinase.

Metabolism of Other Hexoses and Related Compounds

Fructose and Mannose.—A new type of fructokinase was reported in pea seed homogenates by Medina & Sols (12). This soluble enzyme phosphorylates fructose but not glucose, L-sorbose, mannose, nor 12 other aldo- and ketohexoses tested. This is unlike the liver fructokinase which will phosphorylate other ketohexoses. The primary phosphorylated product of the pea enzyme reaction appears to be fructose-6-phosphate.

Another specific fructokinase leading to fructose-6-phosphate has been identified by Doudoroff *et al.* (146) in fructose-adapted strains of *Pseudomonas saccharophila.* These cultures also utilized mannose. This aldohexose was converted to fructose by a mannose isomerase (147) and further metabolized by the 2-keto-3-deoxy-6-phosphogluconate pathway. This fructose-adapted *Pseudomonas* also phosphorylated mannose to mannose-6-phosphate, but no further utilization of the ester was detected. One possible reaction of mannose-6-phosphate is its conversion to mannose-1-phosphate. Further metabolism of the hemiacetal compound has been described by Munch-Peterson (148) for extracts of brewer's yeast which contain a GDP-mannose pyrophosphorylase which catalyzes a reversible pyrophosphorylation of GDP-mannose into GTP and mannose-1-phosphate. It is tempting to speculate that the nucleotide-linked mannose acts as the mannolyl donor in the synthesis of mannose-containing polysaccharides.

Galactose.—According to Hassid *et al.* (149), the utilization of this hexose by tissues of higher plants appears to occur by a mechanism similar to that observed in animal tissues and galactose-adapted strains of yeast. Galactose was converted to galactose-1-phosphate in *Canna* leaves indicating a galactokinase. The synthesis of essentially glucose-1-C^{14} from galactose-1-C^{14} in wheat seedlings indicated an interconversion not involving a prior degradation of the chain. The isolation of UDPGal from mung bean seedlings by Ginsburg *et al.* (150) suggests that the conversion of galactose to glucose is linked to the metabolism of uridine nucleotides and may involve:

$$\text{Galactose-1-phosphate} + \text{UDPG} \rightleftharpoons \text{UDPGal} + \text{glucose-1-phosphate} \quad 16.$$

$$\text{UDPGal} \rightleftharpoons \text{UDPG} \quad 17.$$

$$\text{UDPG} + \text{PP} \rightleftharpoons \text{UTP} + \text{glucose-1-phosphate} \quad 18.$$

$$\text{UDPGal} + \text{PP} \rightleftharpoons \text{UTP} + \text{galactose-1-phosphate} \quad 19.$$

The enzymes have been called, respectively, galactose-1-phosphate uridyl transferase, UDP-4-epimerase (galactowaldenase), UDPG pyrophosphorylase, and UDPGal pyrophosphorylase.

A series of elegant papers by Hassid and his students has described the presence of the enzymes catalyzing the reactions of equations 17, 18, and 19 in higher plants. The UDPGal-4-epimerase was detected by Neufeld *et al.* (151) in the 50 to 75 per cent ammonium sulfate fraction of mung bean seedlings. It was not reported whether the plant enzyme is similar to the mammalian enzyme [Maxwell (152)]. It requires catalytic amounts of DPN for activity. These same extracts also carried out a similar epimerase reac-

tion involving xylose and arabinose. It is not clear, however, whether the UDPGal-4-epimerase or a UDP-pentose-4-epimerase is involved. A similar preparation was shown by Ginsburg (153) to contain an enzyme specifically catalyzing the pyrophosphorylation of UDPG. The UDPGal pyrophosphorylase was found to be widespread among plants (151). These authors (151) also described in mung bean seedlings the general system

$$\text{UTP} + \text{sugar-1-phosphate} \rightleftharpoons \text{UDP-sugar} + \text{PP} \qquad 20.$$

where the sugar may be, in addition to α-D-galactose, β-L-arabinose, α-L-arabinose, or α-D-xylose.

Galactose-1-phosphate uridyl transferase and UDPGal-4-epimerase together with glucose-6-phosphate dehydrogenase can be used to provide a specific assay method for α-galactose-1-phosphate (154).

According to DeLey & Doudoroff (155), *Pseudomonas saccharophila* oxidizes galactose by a pathway not involving galactokinase and the 4-epimerase. The pathway includes: (*a*) the DPN-linked oxidation of galactose to galactono-γ-lactone; (*b*) the hydrolysis of the lactone to galactonic acid; (*c*) the dehydration of galactonic acid to 2-keto-3-deoxygalactonic acid; and (*d*) the reaction of the keto acid with ATP to yield pyruvic acid and D-glyceraldehyde-3-phosphate.

Hexitols.—D-Mannitol, the common reduction product of D-mannose and D-fructose, has been shown to be widespread in higher plants but little is known of its mode of formation in these tissues. Working with the brown marine alga, *Fucus vesiculosus*, Bidwell *et al.* (156) demonstrated that 95 per cent of the soluble activity of incorporated photosynthetic $C^{14}O_2$ entered D-mannitol. On the other hand, the main product of photosynthesis in the red algae was a glycerol mannoside (157). The enzyme, D-mannitol-1-phosphate dehydrogenase, a soluble enzyme isolated from *Escherichia coli* by Wolff & Kaplan (158), reduces fructose-6-phosphate reversibly to mannitol-1-phosphate in the presence of DPNH and may also be responsible in higher plants for the link between the EMP pathway and mannitol. Since the reaction has an absolute specificity for fructose-6-phosphate and an equilibrium far in favor of mannitol phosphate, it can serve as an assay for fructose-6-phosphate.

The oxidation of D-sorbitol by soluble extracts of *Acetobacter suboxydans* has been observed by Cummins *et al.* (159) to proceed by one of two pathways, depending upon which pyridine nucleotide is present. In the presence of TPN, sorbose is formed; in the presence of DPN, fructose is formed. This system is analogous to the xylitol conversion to D- and L-xylulose whereby the oxidation of a sugar alcohol can occur at different ends of the molecule by DPN- and TPN-specific dehydrogenases. Previously this group had demonstrated that a particulate fraction of the same organism catalyzed a one-step oxidation of sorbitol [Widmer *et al.* (160)].

Glucosamine.—A most significant discovery in recent years has been the realization of the key role of uridine derivatives of hexoses in the interconversions of hexoses and the synthesis of various disaccharides and poly-

saccharides. Newer evidence now strongly suggests that the uridine derivatives of the amino sugars serve as the building blocks in the synthesis of polysaccharides of which chitin is an example. The various reactions to be discussed in this section are summarized in Figure 8.

The synthesis of D-glucosamine-6-phosphate from D-hexose-6-phosphate and L-glutamine was first demonstrated in *Neurospora crassa* by Leloir & Cardini (161). Since then, a closer study of the hexose phosphate involved has given contradictory results. Using the *N. crassa* enzyme, Blumenthal *et al.* (162) found the precursor of D-glucosamine-6-phosphate to be fructose-6-phosphate, whereas in rat liver extracts glucose-6-phosphate appears to be the more effective substrate [Pogell & Gryder (163)]. In this same work, the interesting observation was made that glucose-6-phosphate stabilized the very labile rat liver enzyme to a high degree. It may well be that a common intermediate is formed which is the immediate hexose precursor. Glucosamine-6-phosphate has been also synthesized by phosphorylation with ATP (164) and by reaction between fructose-6-phosphate and NH_4^+ (165). The deamination of glucosamine-6-phosphate to fructose-6-phosphate and ammonia has been observed in extracts of *Aerobacter cloacae* (166), hog kidney (165, 167), and *Escherichia coli* (167). The latter group could not confirm the speculation of Leloir & Cardini (165) that N-acetylglucosamine-6-phosphate is an intermediate in the reaction.

Davidson *et al.* (168) have described an enzyme capable of acetylating glucosamine-6-phosphate in the presence of acetyl CoA in extracts of *Neurospora crassa*, *Penicillium* species, and various animal tissues. The purified *Neurospora* enzyme is inactive with glucosamine, galactosamine, *p*-nitroaniline, or amino acids as substrates. Cell-free extracts of a variety of bacteria contain an enzyme hydrolyzing N-acetylglucosamine to glucosamine and acetate (169).

An enzyme system from *N. crassa* which catalyzes the reversible conversion of N-acetylglucosamine-6-phosphate to the 1-phosphate has been characterized by Reissig (170). The enzyme requires Mg^{++} and a complex-forming agent in addition to a diphosphate cofactor like N-acetylglucosamine-diphosphate or glucose-1,6-diphosphate. Equilibrium was reached when the mixture contained 86 per cent of the 6-phosphate component.

The link between the amino sugars and their conversion into polysaccharides appears to be their UDP derivatives. Uridine diphosphate N-acetylglucosamine found in mung bean seedlings by Solms & Hassid (171) is probably synthesized from N-acetylglucosamine-1-phosphate and UTP as demonstrated by Maley & Lardy (172) in liver homogenates. The latter workers have shown that similarly, with glucosamine-1-phosphate, uridine diphosphoglucosamine is formed by rat liver nuclei. UDP N-acetylglucosamine can also act as a glycosyl donor in the synthesis of insoluble chitin with extracts of *Neurospora crassa* [Glaser & Brown (173)]. The enzyme, termed "chitin synthetase," is freely reversible but needs a preformed chitodextrin as primer. Since the investigators used UDP N-acetylglucosamine synthesized by yeast which probably contains α-glycosidically-bound acetylglucosamine, "chitin synthetase" apparently proceeds with inversion since chitin

found in nature consists of units of N-acetyl-D-glucosamine joined to one another by means of β-1,4-glycosidic bonds.

Another reaction of UDP acetylglucosamine discovered by Cardini & Leloir (174) involves inversion at C_4, similar to the galactowaldenase reaction, with the synthesis of UDP acetylgalactosamine. The same preparations of rat and guinea pig liver could also break down UDP acetylglucosamine into free acetylgalactosamine, uridine monophosphate, and inorganic phosphate.

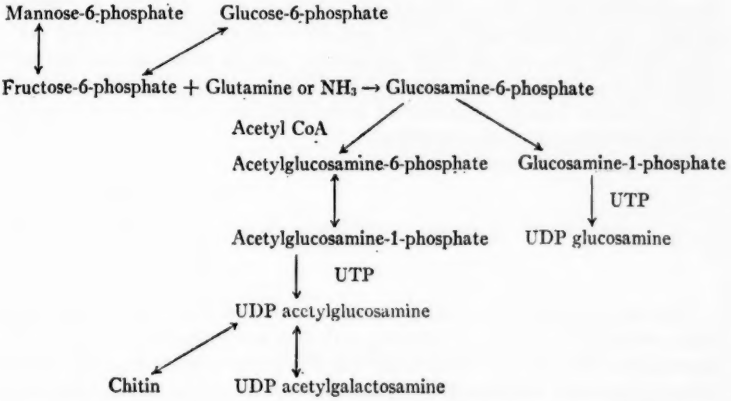


FIG. 8. Relation between the EMP pathway and hexosamine metabolism.

Glucuronic acid.—The relation between D-glucuronic acid, a basic unit of plant polysaccharides ("hemicellulose"), and the hexoses of the EMP pathway has now become clear.

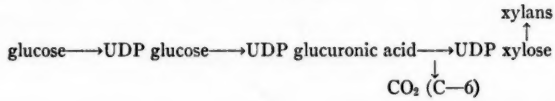
Strominger & Mapson (126) have shown that the soluble fraction of a calf liver homogenate contains an enzyme, UDP dehydrogenase, which catalyzes a two-step oxidation of UDPG to uridine diphosphoglucuronic acid:

$$\text{UDPG} + 2\ \text{DPN}^+ \rightarrow \text{UDP glucuronic acid} + 2\ \text{DPNH} + 2\ \text{H}^+ \qquad 21.$$

A further 200- to 400-fold purification failed to resolve the irreversible oxidation into two steps. Strominger & Mapson (126) have purified this enzyme about 1000-fold from an extract of germinated pea seedlings. Like its counterpart in liver, the purified enzyme catalyzes a two-step oxidation of UDPG but does not oxidize UDPGal. On the other hand, crude preparations of the enzyme oxidized UDPGal at the same rate as UDPG, but the nucleotide product contained only glucuronic acid indicating the presence of a galactowaldenase enzyme. Alternatively, the possibility exists that a separate dehydrogenase exists which can oxidize UDPGal directly. By analogy to the oxidation of UDPG, a separate and specific dehydrogenase of UDPGal is indicated. Slater & Beevers (176) have found that D-glucuronic acid-6-C^{14} and D-glucurono-γ-lactone-C^{14} can serve as respiratory substrates for corn coleoptiles. While both yielded $C^{14}O_2$, only the latter compound placed tracer

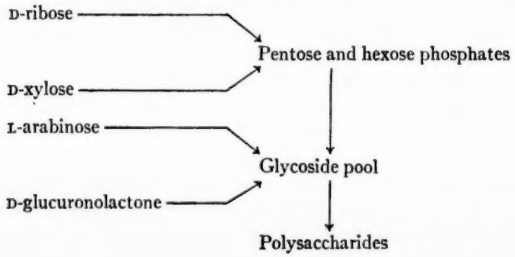
in the insolubles which consisted mainly of C^{14}-xylose. Whereas attempts to demonstrate an enzyme in the coleoptile extracts capable of hydrolyzing the lactone to the acid were unsuccessful (176), Eisenberg & Field (177) were able to detect a hydrolyzing enzyme in rat liver preparations. The absence of radioactivity in the nonnucleotide-bound pentose, coupled with the high level of C^{14} in the insolubles, suggests that free glucuronic acid is not decarboxylated by the corn coleoptile preparations. These data with the corn coleoptile, together with the findings of Rabinowitz & Sall (178) that the decarboxylation of glucuronolactone and glucuronic acid by rat kidney preparations is stimulated by UTP and the work of Solms & Hassid (171) revealing the presence of UDP glucuronic acid in mung beans, strongly supports the mechanism of xylan synthesis proposed by Altermatt & Neish (179), namely, that the synthesis of xylan units occurs by a process not involving D-xylose as an intermediate.

This mechanism involves the following:



22.

Enzyme evidence concerning the direct conversion of D-glucuronic acid to its uridyl derivative is still lacking although the tracer data suggests this possibility (179). In this connection, it is of interest to note that rat kidney preparations can degrade UDP glucuronic acid to free glucuronic acid, uridine, and inorganic phosphate with transient formation of small amounts of glucuronic acid-1-phosphate and uridine monophosphate (180). On the other hand, the labeling patterns of D-xylose derived from xylans of wheat plants (140, 179, 181, 182) incubated with specifically labeled D-xylose and D-ribose demonstrates the inability of tissues to make direct use of these pentoses in pentosan synthesis. The data show that D-xylose is first converted to hexose phosphate by a transaldolase-transketolase sequence of reactions before being utilized for wheat polysaccharides. In contrast to D-xylose and D-ribose, the incorporation of L-arabinose-1-C^{14} into free xylose (138) or xylan (183) occurs with little rearrangement of the carbon skeleton. To account for the differences in labeling data, the following tentative outline of polysaccharide synthesis has been proposed (183):

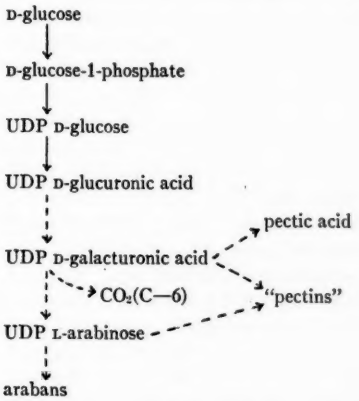


23.

The L-arabinose tracer pattern would suggest a conversion of L-arabinose to UDP arabinose which under the influence of a 4-epimerase is converted to UDP xylose (151). An enzymatic pathway of this type would explain the tracer patterns provided a means of converting arabinose to arabinose-1-phosphate were known. At present, evidence is also lacking for the ability of UDP xylose to act as a xylosyl donor for xylan synthesis. A second pathway for L-arabinose metabolism might occur through L-ribulose. This pathway, elucidated in L-arabinose-adapted bacteria, is discussed in another section. However, L-ribulose has not been detected in higher plants.

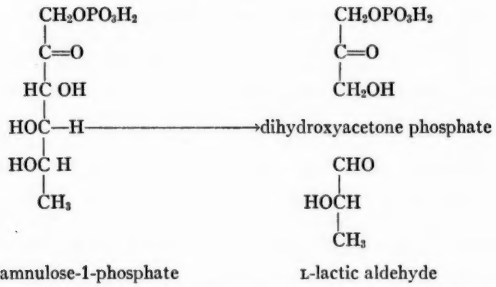
Galacturonic acid.—Seegmiller *et al.* (184) have published a complete report of their studies on the conversion of glucose-1-C^{14} into the "pectins" of the boysenberry. Their results suggest that glucose is converted to the galacturonic acid and arabinose moieties without cleavage of the carbon chain.

Their tracer data are in harmony with the following possible sequence:



24.

6-Deoxy-hexoses.—L-Rhamnose, 6-deoxy-L-mannose, L-fucose, and 6-deoxy-L-galactose have been shown to occur in many materials including cell walls of algae, gums, bacterial polysaccharides, and in the free state in the leaves and blossoms of the poison ivy, *Rhus toxicodendron.* Although these methylpentoses have been known for some time, only recently has an elucidation of their biosynthesis begun. Using *Escherichia coli* adapted to L-rhamnose, Tecce & Di Girolamo (185), Wilson & Ajl (186), and Englesberg (187) have shown that the initial step in the catabolism of L-rhamnose is its isomerization to L-rhamnulose followed by a conversion of the keto sugar to L-rhamnulose-1-phosphate by a kinase reaction with ATP (188). The 1-position is indicated for the phosphate because of the ease of hydrolysis of the ester. Further metabolism of L-rhamnulose-1-phosphate is unknown, but the isolation of propanediol (189) and lactic aldehyde (190) as fermentation products of rhamnose are indicative of a cleavage into two 3-carbon compounds. Utter (125) has proposed the following aldolase-type reaction:



25.

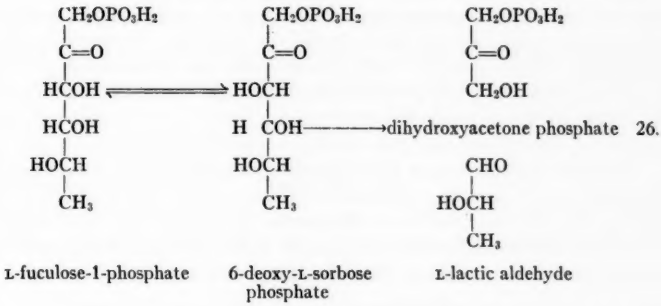
L-rhamnulose-1-phosphate L-lactic aldehyde

Hauser & Karnovsky (191) have investigated the synthesis of rhamnose by using cultures of *Pseudomonas aeruginosa* which produce a rhamnolipide composed of two moles of L-rhamnose and two moles of β-hydroxydecanoic acid. Using this system, they demonstrated that glycerol can furnish all of the carbon of the rhamnolipide but that acetate can supply only the β-hydroxydecanoic acid. It was further shown that DL-glycerol-1-C^{14} yielded rhamnose labeled in carbon atoms 1, 3, 4, and 6, while DL-glycerol-2-C^{14} placed tracer in carbon atoms 2 and 5 (192). A later paper (193) revealed that propanediol or fructose could also serve as the sole precursor of rhamnose. When D-fructose-6-C^{14} was the carbon source, rhamnose was labeled almost exlusively in C-6; however, tracer amounts of D-glucose-6-C^{14} added to glycerol media yielded rhamnose labeled in both C-1 and C-6, with the latter carbon atoms somewhat more active. Extracts of the organism contain an active DPN-linked dehydrogenase capable of attacking propanediol. The oxidation product was not identified.

Sandman & Miller (194, 195) have shown that the reduction of lactic aldehyde by DPNH can be catalyzed by yeast alcohol dehydrogenase; however, the reverse reaction did not occur even at relatively high enzyme concentrations. In addition, the yeast aldehyde dehydrogenase-DPN system was able to oxidize lactic aldehyde to lactic acid although the reverse reaction did not occur.

The initial step in L-fucose metabolism is its conversion to the keto-sugar, L-fuculose [Green & Cohen (196)]. According to Huang & Miller (197), L-fuculose is converted to methylpentose phosphate when incubated with an extract of *Escherichia coli* and ATP. The assumption is made that the phosphate is attached to carbon atom 1. A phosphoketoepimerase, prepared from an extract of *Lactobacillus pentosus*, appeared to convert L-fuculose phosphate to 6-deoxy-L-sorbose phosphate (see fig., top of page 367).

It was postulated that the latter compound cleaved to dihydroxyacetone phosphate and L-lactic aldehyde. This proposal is incompatible with the tracer data obtained with strains of *Aerobacter cloacae* which produce a polysaccharide composed of approximately equal amounts of L-fucose, D-glucose, D-galactose, and uronic acid (198). Since this bacterium converted D-glucose-1-C^{14} to L-fucose-1-C^{14}, and D-glucose-6-C^{14} to L-fucose-6-C^{14}, it



26.

L-fuculose-1-phosphate | 6-deoxy-L-sorbose phosphate | L-lactic aldehyde

would appear that the hexose is converted to a methyl pentose either directly without cleavage of the carbon chain or via a pathway involving the cleavage of the glucose carbon chain to fragments which are not in equilibrium with each other and which are subsequently recombined to form L-fucose (199, 200). These data are, therefore, similar to those obtained with *Pseudomonas aeruginosa* (193) for L-rhamnose with D-fructose-6-C^{14} as substrate but different from those with D-glucose-6-C^{14} in the presence of glycerol. The synthesis of the 6-deoxy-hexoses in higher plants has received no attention.

Metabolism of Other Glycosyl Compounds

Cellulose.—The data of Greathouse (201) with intact *Acetobacter xylinum* and Brown & Neish (202) with wheat plants show that glucose is converted to the polysaccharide with little or no degradation and recombination of the hexose. A similar finding was reported by van Sumere & Shu (203) for *Aspergillus niger*. In this same investigation, the interesting observation was made that D-xylose and D-ribose were converted to mycelial glucosan by different pathways. In the glucose derived from xylose-1-C^{14}, about 50 per cent and 25 per cent of the C^{14} was found in carbon positions 1 and 3 respectively, while the activities of carbon positions 4, 5, and 6 were low. On the other hand, ribose-1-C^{14} gave a glucose with almost equal activity in carbon atoms 1 and 6 and fair activity in C-3. A conventional transaldolase-transketolase sequence could explain most of the pattern in the glucose derived from xylose. It was proposed that ribose-1-C^{14} follow an additional pathway in which the pentose molecule is inverted by the formation of a pentose alcohol with a subsequent oxidation at position 5. The following pathway was proposed:

$$\text{D-ribose} \rightarrow \text{D-ribulose} \rightarrow \text{D-arabitol} \rightarrow \text{D-xylulose} \qquad 27.$$

Enzyme studies with extracts of *Acetobacter xylinum* (204, 205) have shown that ATP is needed for the conversion of glucose to polymerized glucose, while Hestrin & Schramm (206) have noted that nonviable lyophilized

preparations of the same organism need oxygen to carry out this conversion. These data would indicate that phosphate esters are involved in cellulose formation in the organism (207). Glaser (208) has obtained a particulate enzyme preparation from sonic extracts of *A. xylinum* which can incorporate the C^{14} of UDPG-C^{14} into celluose in the presence of a soluble cellodextrin as primer. The reverse reaction could not be demonstrated.

Trehalose.—Trehalose, a disaccharide consisting of two glucose units of pyranose structure with a glycosidic linkage connecting the two isomeric carbons, is found mainly in the fungi, yeast, and the "resurrection plant," *Selaginella lepidophylla.* Cabib & Leloir (209) have partially purified an enzyme from brewer's yeast which catalyzes the reaction

$$\text{UDPG} + \text{glucose-6-phosphate} \rightarrow \text{UDP} + \text{trehalose phosphate.} \qquad 28.$$

The rotary power of the reaction product indicated a 1,1-α-glucose-α-glucoside, the naturally-occurring form of the disaccharide. The equilibrium of the reaction favors trehalose phosphate with a minimal K value of 50. An upper limit of -4.4 kcal. for the $\Delta F°$ of hydrolysis of the trehalose glycosidic bond was calculated which is similar to a value of -4 kcal. for the glycosidic linkage of maltose (210). These values contrast with a $\Delta F°$ of -5.75 kcal. for the glycosidic bond of sucrose calculated on the assumption that the $\Delta F°$ of hydrolysis of sucrose is -6.6 kcal. (211) with a correction of -0.85 kcal. for the partial transformation of the liberated fructofuranose into fructopyranose. The purified yeast preparation also contains a specific phosphatase for trehalose phosphate.

Sucrose.—Contrary to previous negative reports (212), a sucrose phosphorylase has been claimed in sugar cane leaves. The enzyme was partially purified by ammonium sulfate fractionation (30 to 60 per cent), followed by a calcium phosphate gel step. On incubating glucose-1-phosphate and fructose with the purified fraction, a disappearance of fructose occurred with a concomitant release of inorganic phosphate. The possibility that this extract still contained sufficient uridine nucleotides to form uridyl compounds was not tested. Cardini *et al.* (213) and Leloir & Cardini (214) were the first to demonstrate that plant tissue contains two mechanisms for synthesizing sucrose, one involving a glycosyl transfer from UDPG to fructose-6-phosphate and the other a similar glycosyl transfer to fructose. More recent evidence shows that, while extracts of sugar beet (215) and *Impatiens holstii* (216) have mechanisms apparently specific for fructose-6-phosphate, pea extracts can catalyze a glycosyl transfer from UDPG to either the free ketohexose or its ester (217, 218). Indeed, the latter group has reported that L-sorbose, D-xylulose, and D-rhamnulose can accept a D-glucose moiety to form D-glucosyl-L-sorboside, D-glucosyl-D-xyluloside, and D-glucosyl-D-rhamnuloside, respectively.

The probable sequence of reactions in the synthesis of sucrose may be summarized by the following reactions:

$$ATP + UDP \leftrightarrow UTP + ADP \quad \text{nucleoside diphosphate kinase} \qquad 29.$$

$$UTP + \alpha\text{-D-glucose-1-phosphate} \leftrightarrow UDPG + PP \quad \text{UDP-glucose pyrophosphorylase} \qquad 30.$$

$$UDPG + \text{D-fructose-6-phosphate} \rightarrow \text{sucrose phosphate} + UDP$$

UDPG-fructose-6-phosphate transglycosylase 31.

$$UDPG + \text{D-fructose} \rightarrow \text{sucrose} + UDP \quad \text{UDPG-fructose transglycosylase} \qquad 32.$$

$$\text{Sucrose phosphate} \rightarrow \text{sucrose} + P_i \quad \text{sucrose phosphatase} \qquad 33.$$

Lactose.—According to our present knowledge, derived mainly from enzyme studies with mammalian tissues, the transformation from glucose to lactose may be considered to occur as follows:

$$\text{glucose} + ATP \rightarrow \text{glucose-6-phosphate} + ADP \qquad 34.$$

$$\text{glucose-6-phosphate} \leftrightharpoons \text{glucose-1-phosphate} \qquad 35.$$

$$\text{glucose-1-phosphate} + UTP \rightleftharpoons UDPG + PP \qquad 36.$$

$$UDPG \rightleftharpoons UDPGal \qquad 37.$$

$$UDPGal + \text{glucose-1-phosphate} \rightarrow \text{lactose-1-phosphate} + UDP \qquad 38.$$

$$\text{lactose-1-phosphate} \rightarrow \text{lactose} + P_i \qquad 39.$$

Reactions 34 and 37 have been dealt with elsewhere in this review. Gander *et al.* (219) have presented evidence for the galactosyl transferase (reaction 38) and the phosphatase (reaction 39) in mammary gland extracts.

Clearly, in this mechanism the two hexose moieties of the lactose would be expected to possess equal labeling and similar C^{14} distribution patterns since glucose-1-phosphate is the precursor of both glucose and galactose. The tracer experiments carried out to test this mechanism have yielded varied results. Dimant *et al.* (220) have synthesized lactose which contained nearly equal amounts of radioactivity in the galactose and glucose moieties in a bovine mammary gland perfused with blood containing glucose-1-C^{14}. The tracer was located almost exclusively in C-1 of each of the monosaccharides, indicating that the conversion of glucose to galactose does not involve a rearrangement of the carbon chain and that lactose is synthesized by a simple union of the two monosaccharides. A similar result was obtained with lactating goats (221). In contrast to these data are those of Wood & his co-workers. When milk lactose was obtained from cows which were given intravenous acetate-1-C^{14}, acetate-2-C^{14}, or $NaHC^{14}O_3$, the glucose and galactose moieties of lactose contained approximately equal activities; however, with acetate-1-C^{14} and $NaHC^{14}O_3$, the C-4 of both hexoses contained the highest activity, while with acetate-2-C^{14} both sugars were similarly and uniformly labeled (222). In addition, when the synthesis of milk lactose was studied by perfusion of isolated cow udders with acetate-1-C^{14} or propionate-1-C^{14}, about 90 per cent of the label incorporated into the disaccharide was found in the galactose with C-4 containing about three times as much C^{14} as C-3 [Wood *et al.* (223)]. A tentative explanation offered

by Wood *et al.* (224) for this asymmetrical distribution of tracer in galactose is a slow triose phosphate isomerase with a dilution of the upper portion of hexose by an unlabeled triose like glycerol; however, a more recent note has not supported this reasoning (225).

The mechanism of lactose synthesis in higher plants has lagged because a convenient plant tissue has not been available. A preliminary paper by Venkataraman & Reithel (226) indicates that *Achras zapota* might furnish the proper tissue.

The Tricarboxylic Acid Cycle

Occurrence.—All of the enzymes and organic acids of the citric acid cycle have been demonstrated in plants. The assumption that the individual enzymes can operate as a unit is supported by many papers describing the oxidative characteristics of mitochondria isolated from various plant sources and by isotope experiments. These qualitative observations leave little doubt as to the occurrence of a Krebs citric acid cycle tightly bound to plant mitochondria; however, they present only suggestive evidence that the cycle plays a major role as a terminal oxidative mechanism in the tissues of higher plants. A critical evaluation of the extent of metabolism over this pathway in higher plants has not appeared.

Quantitative significance.—One of the more interesting isotope experiments was performed by Drummond & Burris (227) with lupine mitochondria. They added to a mitochondrial preparation 200 μmoles each of pyruvic acid-2-C^{14}, malic acid as "sparker," and another citric acid cycle acid to serve as a trapping acid. Analysis showed that every acid of the citric acid cycle accumulated radiocarbon. These workers concluded that each acid participated in the cyclic oxidation of pyruvic acid. An essentially similar specific activity between pyruvic acid and citric acid was further evidence of a conventional cycle in these mitochondria. Specific activity data, unfortunately, were not reported for the other acids.

Acetate-grown resting cells of the fungus, *Penicillium chrysogenum*, oxidize specifically-labeled acetate by a tricarboxylic acid cycle as shown by the labeling pattern in citrate and glutamate [Goldschmidt (228)]. In the same work, glutamic acid could be detected on chromatograms after less than five seconds of exposure to radioactive acetate whereas α-ketoglutarate was still unlabeled. Because of a large endogenous pool, it was estimated that from 2 to 15 min. was required before labeled citrate and glutamate was formed from the C^{14}-acetate which showed a distribution of radioactivity predictable on the basis that the cycle had completed two or more turns. Cultures of the fungus, *Zygorrhynchus moelleri*, when grown on unlabeled glucose in the presence of $C^{14}O_2$ or C^{14}-acetate, showed a labeling pattern in glutamate and aspartate in agreement with a citric acid cycle (229). The distribution of tracer in phosphoglyceric acid formed from glycolic acid-C^{14} by *Scenedesmus*, Strain D_3 (Gaffron), can be explained by the mediation of a conventional Krebs cycle in this alga [Schou *et al.* (230)].

Glutamic acid isolated from the gluten of wheat plants to which acetate-1-C^{14} or -2-C^{14} was administered during growth were degraded by Bilinski & McConnell (231). Sixty-three per cent of the activity of the glutamic acid arising from acetate-1-C^{14} was in C-5 and 20 per cent in C-1; glutamic acid from acetate-2-C^{14} contained 43 per cent of the activity in C-4 and about 18 per cent in each of carbon atoms 2 and 3. The distribution results show that the citric acid cycle provides a major pathway for the biosynthesis of the dicarboxylic amino acids of wheat gluten. On the other hand, Nelson & Krotkov (232) partially degraded glutamic acid isolated from the leaves of the broad bean incubated with acetate-1-C^{14} and, finding only 13 per cent of its activity in position 1, concluded that the citric acid cycle primarily could not account for the formation of this amino acid. The two data can probably best be explained by a difference in the rate of the operation of the citric acid cycle. In a continuation of their studies on wheat plants, Bilinski & McConnell (233) observed that administered pyruvic acid-2-C^{14} gives a labeling pattern in glutamic acid similar to that of acetate-1-C^{14}, indicating entry of the keto acid into the citric acid cycle after decarboxylation. The most active carbon atom of alanine derived from pyruvate-2-C^{14} was carbon atom 1. If pyruvate-2-C^{14} were converted to a two carbon unit which enters the glyoxylate cycle then the resultant malate-C^{14}, after equilibrating with fumarate, would give rise to pyruvic acid-1-C^{14} and after transamination, alanine-1-C^{14} [Neal & Beevers (234)].

Using the methods first employed by Swim & Krampitz (235) with *Escherichia coli* and by Saz & Krampitz (236) with *Micrococcus lysodeikticus*, DeMoss & Swim (237) have given convincing evidence that the tricarboxylic acid cycle is a major pathway for acetate oxidation by nonproliferating cells of baker's yeast. Their analytical data demonstrate isotopic equilibrium between the original acetate and citrate, α-ketoglutarate, succinate, fumarate, malate, and glutamate. The distribution of C^{14} in citrate and succinate was in accordance with the cycle. A similar specific activity of the carboxyls of citrate and CO_2 indicates that a pathway other than the conventional cycle is not operating. It should be noted, however, that these data do not exclude the possibility that acetate may be metabolized to a limited extent by the oxidative condensation of succinate (Thunberg reaction).

Metabolic Systems Involving Acids of the Citric Acid Cycle

Thunberg-Wieland Cycle.—This dicarboxylic acid cycle, whose key reaction is the condensation of two C_2 units (acetate) to form the C_4 dicarboxylic acid, succinic acid, has been invoked at intervals in the biochemical literature to account for the ability of various bacteria, algae, and fungi to grow on acetate as the sole source of carbon (238, 239). The weak point of the cycle thus far has been the lack of enzymatic evidence for this condensation. However, the detection of a succinate-cleaving enzyme in a heavy particle fraction of *Tetrahymena pyriformis* and in homogenates of rat brain, heart, liver, and skeletal muscle by Seaman & Naschke (240) strongly sug-

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gests that a dicarboxylic acid cycle first suggested by Thunberg and Wieland may play a major role in organic acid metabolism under certain conditions. The enzyme which has been purified from extracts of *T. pyriformis* (241) about 150-fold by ammonium sulfate and acetone fractionation followed by treatment with calcium phosphate gel shows an almost absolute requirement for succinate, acetyl CoA, ATP, ADP, P_i, DPN, and a divalent metal (Mg^{++} or Mn^{++}). This enzyme, which catalyzes the reaction

$$\text{succinate} + 2\ \text{ATP} + 2\ \text{CoA} + \text{DPNH} + H^+ \rightleftharpoons 2\ \text{acetyl CoA} + 2\ \text{ADP} + 2\ P_i + \text{DPN}^+ \qquad 40.$$

was assayed by measuring the formation of acetylhydroxamic acid in the presence of malonate to block succinate oxidation and arsenite to block pyruvate oxidation. The apparent equilibrium constant of the reaction, which markedly favors acetyl CoA formation, was found to be 2.83×10^{-6} at pH 8.4. The formation of $C^{14}H_3\,COOH$ from methylene-labeled C^{14} succinate and $CH_3C^{14}OOH$ from carboxy-labeled succinate was given as further proof of a central cleavage of succinate, since a conversion of succinate to a C_2 unit by the conventional cycle would have produced other results. Further purification will determine whether one enzyme can catalyze a reaction which apparently includes many steps. A preliminary study by Davies (242) indicates that a similar enzyme is present in pig heart muscle; however, in this work the presence of the conventional citric acid cannot be ruled out.

The "glyoxylate cycle."—Isocitritase and malate synthetase, newly discovered enzymes, catalyze two reactions involving glyoxylate in a pathway which has been called the "glyoxylate by-pass" (243). In conjunction with known reactions leading from citrate to isocitrate and from malate to oxalacetate, it can apparently represent a variant of the tricarboxylic acid cycle of Krebs and the dicarboxylic acid cycle of Thunberg and Wieland. Whereas the net effect of the conventional citric acid cycle is the combustion of one mole of acetate to two moles of CO_2, the operation of this new system, like the Thunberg-Wieland cycle, leads to the net synthesis of one mole of a C_4 dicarboxylic acid from two moles of acetate. The "glyoxylate by-pass," together with part of the tricarboxylic acid cycle, has been called the "glyoxylate cycle" and has been proposed as a mechanism for the synthesis of dicarboxylic acids from acetate (244, 245). This cycle, which is outlined in Figure 9, shows an over-all reaction of 2 acetate→succinate +2H.

This metabolic cycle was initiated by the demonstration that crude extracts of *Pseudomonas aeruginosa* could convert various citric acid cycle acids to glyoxylate and succinate [Campbell *et al.* (246)]. The enzyme has also been detected in a wide variety of aerobic and facultative bacteria (247, 248), in various fungi (249), in *Escherichia coli* (250), and in a wide variety of plants tissues, including pumpkin, cottonseed, peanuts, flax, sesame, soybean, watermelon, sunflower, castor bean (251), and germinating marrow seed (252).

The aldol condensation catalyzed by isocitritase

$$\text{glyoxylate} + \text{succinate} \rightleftharpoons \text{L}\,(+) - \text{isocitrate} \qquad 41.$$

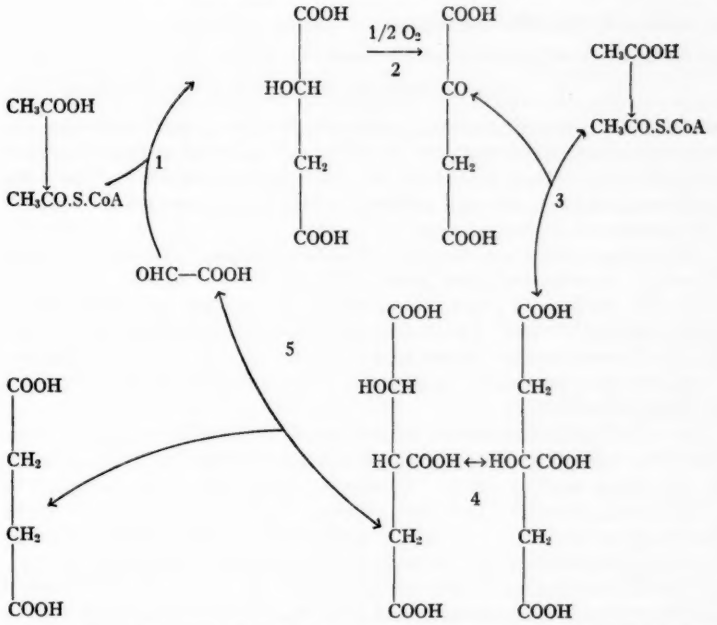


FIG. 9. Reactions of the glyoxylic acid cycle (glyoxylate shunt). The individual reactions are catalyzed by the following enzymes: 1. malate synthetase; 2. malic dehydrogenase; 3. citric acid condensing enzyme; 4. aconitase; 5. isocitritase.

occurs with a negative free energy (−2.1 kcal., pH 7.6) in the direction of condensation (253) and therefore resembles the citritase reaction. The *P. aeruginosa* enzyme (253), as well as the plant enzyme (251), requires Mg^{++} and cysteine or glutathione as activators.

The second enzyme involving glyoxylate was first reported by Wong & Ajl (254) in *E. coli* and called malate synthetase.

$$H_2O + \text{glyoxylate} + \text{acetyl CoA} \rightarrow \text{malate} + \text{CoA} \qquad 42.$$

This condenstation reaction is presumably similar to the citric acid condensing enzyme and represents the hydrolysis of a thio ester as the driving force. This would suggest a reaction strongly favoring synthesis and a ΔF of -7 kcal. (255). The reaction has also been demonstrated in extracts of *Pseudomonas fluorescens* (256) and in the cell-free supernatant fraction of castor bean (257). Enzymatic and tracer evidence has been presented to establish the validity of the hypothesis that the glyoxylate cycle can account for the conversion of acetyl CoA to carbohydrate in the four to six day castor bean seedling (257, 258). The synthesis of carbohydrate from fat was visualized

to proceed by the following stages:

fat → acetyl CoA → (glyoxylate cycle) → malate →

phosphoenolpyruvate → EMP pathway → carbohydrate 43.

While the activity of aconitase, malic dehydrogenase, and isocitritase was sufficient to account for the required 25 micromoles of carbohydrate (expressed as hexose) per hour from fat, the apparent activities of the citric acid condensing enzyme and malate synthetase were only of the order of one per cent of the desired rate.

It is important to note that isocitritase and malate synthetase have been found only in microorganisms grown on acetate as the sole source of carbon (245, 256, 258) or in plant tissues which are actively converting fat to carbohydrate (257, 259). The inability of Madsen (260) to demonstrate these enzymes in mammalian tissues, coupled with the appearance of these enzymes only in certain cells, would suggest that the glyoxylate cycle may be limited in distribution.

In addition to its synthesis by isocitritase, the derivation of glyoxylate, which has been recently isolated by Krupka & Towers (261), from all parts of the wheat seedling except the ungerminated grain is interesting. The flavoprotein, glycolic acid oxidase, which catalyzes the oxidation of glycolic acid to glyoxylic acid, is known in higher plants (262). Zelitch & Ochoa (263) have demonstrated that sodium hydroxymethanesulfonate competitively inhibits the glycolic acid oxidase of spinach leaves. The inhibition is effective when the concentration of the inhibitor is one per cent that of glycolic acid. The compound does not inhibit glyoxylic acid reductase or muscle lactic acid dehydrogenase. In an attempt to explain the inability of the citric acid cycle to operate under high light intensity, Delavan & Benson (264) incubated glycolic acid-C^{14} with isolated spinach chloroplasts and noted that light markedly enhanced $C^{14}O_2$ evolution. Since $C^{14}O_2$ evolution from acetic acid-C^{14} was not stimulated, it was suggested that in the light a shift in substrate and oxidative metabolism occurs in the chloroplast from pyruvate oxidation to glycolic acid and oxidation via glycolic acid oxidase. Glycolic acid could arise from carbon atoms 1 and 2 of xylulose-5-phosphate by a transketolase or phosphoketolase catalyzed reaction, since Griffith & Byerrum (265) have given evidence that ribose-1-C^{14} yields glycine predominantly labeled in the α-carbon position. Glycine may give rise to glyoxylate by transamination (266) or by oxidative deamination (267). The hydrolytic action of allantoinase and allantoic acid on allantoin has been demonstrated in many higher plants to produce glyoxylic acid (268). Although wheat seedlings did not convert exogenous allantoin to glyoxylate, an increase in glyoxylate and a decrease in allantoin in seedlings maintained in nitrogen is suggestive evidence that the wheat plant can catalyze this conversion (269). Studies with *Aspergillus niger* have led to the suggestion that acetate is converted to glycolic and glyoxylic acids (270). More recently, Bolcato & Leggiero (271) have given evidence that glycollate, glyoxylate, and for-

mate are intermediates in the oxidation of acetate to CO_2 by yeast. Isocitritase and the tricarboxylic acid cycle were ruled out as possible sources of glyoxylate since succinate, fumarate, and malate did not give rise to glyoxylate. When *Escherichia coli* was grown on $C^{14}H_3COOH$, serine strongly labeled in the beta carbon was isolated, suggesting that the methyl carbon of acetate was oxidized to a one carbon unit which combined with lycine [Cutinelli *et al.* (272)].

If these observations are confirmed, then, as Utter (125) has pointed out, another dicarboxylic acid oxidative cycle with an over-all reaction of

$$\text{acetate} + 2\ H_2O \rightarrow 2\ CO_2 + 8\ H \qquad 44.$$

is possible.

The carboxylation of acetate.—The fixation of CO_2 by acetate, or a derived C_2 piece to form pyruvic acid, could provide another mechanism for the conversion of fatty acids to carbohydrate and a means of supporting the growth of organisms on acetate as their sole source of carbon. Using the free energy data of Krebs (273), the amount of energy needed to be invested to drive the carboxylation of acetyl CoA can be calculated.

$\text{Pyruvate}^- + 1/2\ O_2 + \text{CoA}^+H^+ \rightarrow \text{acetyl CoA} + CO_2 + H_2O$	− 61,800 cal.	
$DPN^+ + H_2O \rightarrow DPNH + H^+ + 1/2\ O_2$	+ 52,400 cal.	45.
$\text{Pyruvate} + DPN^+ + \text{CoA} \rightarrow \text{acetyl CoA} + CO_2$	− 9,400 cal.	

It would appear that the combination of a good supply of reduced pyridine nucleotide and proper displacing reactions should be possible to reverse this exergonic reaction. The anaerobically-growing photosynthetic bacteria should provide the best test organism. By growing *Rhodospirillum rubrum* anaerobically in the light on $C^{13}H_3C^{14}OOH$ and unlabeled CO_2, followed by isolation and degradation of glycine, serine, alanine, threonine and aspartic acid whose carboxyl carbons were derived from the bicarbonate-carbon and α-carbon atoms of the acetate carboxyl, Cutinelli *et al.* (274, 275) concluded that the contribution of acetate to the carbon skeleton of the amino acids was caused to some extent by a photodirected carboxylation of a C_2 compound derived from acetate. When autotrophically-grown *Chlorella* was incubated under air with acetate-2-C^{14} for two hours in the presence of light at the compensation point, the glucose from starch was shown by Gibbs (276) to have a labeling pattern suggesting that acetate is not metabolized under these conditions by a direct carboxylation. Kornberg & Quayle (277) investigated the effect of CO_2 on the incorporation of acetate-C^{14} by acetate-grown *Pseudomonas fluorescens*. Since the radioactivity of bacterial protein obtained from cells grown on acetate-2-C^{14} and $C^{12}O_2$ was 83 to 85 per cent of that obtained from cells grown on the same isotopic carbon source with a small supply of respired CO_2, they concluded that a process involving the direct carboxylation of acetate or a compound related to it does not play a major role in the synthesis of protein carbon of *P. fluorescens*. Autotrophic

growth and anaerobiosis would appear to be necessary conditions to detect a reaction involving CO_2 fixation with an acetate unit.

Interrelationships of the tricarboxylic acid and dicarboxylic acid cycles.— A number of attempts have been made to establish the relative contribution of the citric acid cycle, the "glyoxylate cycle," and a pathway involving direct carboxylation of a C_2 unit and CO_2 to the metabolism of intact cells. These studies have as their basis the relative isotopic contribution of either the methyl carbon or the carboxyl carbon of acetate to various metabolic products. Either microorganisms grown on isotopic acetate as their sole source of carbon or resting-cell suspensions of unicellular organisms cultured in various ways and then exposed to isotopic acetate have been employed. The same difficulties involved in the use of isotopic techniques for determining a quantitative evaluation of the pentose phosphate and EMP pathways apply. Therefore, the data obtained are always open to doubt. Nevertheless, studies of this type give at least a partial indication of the interrelations between the various pathways whereby most of the energy is derived by the cell during respiration.

Table II gives the expected distribution of tracer in a hexose unit derived from acetate-1-C^{14} or -2-C^{14} metabolized by one of the three routes. The assumption has been made that malate synthesized by malate synthetase is not in complete equilibrium with fumaric acid, prior to its conversion to a hexose unit via oxalacetate and pyruvate. The major difference between the glyoxylate cycle and the citric acid cycle illustrated in Table II is the amount of tracer in C-3 and C-4 of hexose which will also be reflected in the carboxyl carbon of pyruvate.

In an elegant application of this technique, Kornberg & Quayle (277) isolated and degraded alanine and aspartate from acetate-2-C^{14} grown *Pseudomonas fluorescens*. From the observed ratios between the carboxyl carbons and the other carbon atoms of the two amino acids, which were lower than

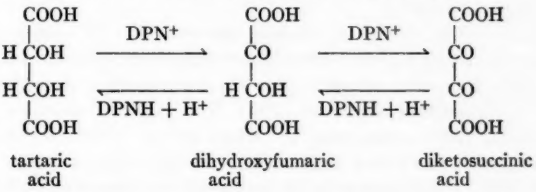
TABLE II

EXPECTED DISTRIBUTION OF C^{14} IN A HEXOSE UNIT DERIVED FROM $C^{14}H_3COOH$ OR $CH_3C^{14}OOH$ BY VARIOUS PATHWAYS

Pathway of metabolism	$C^{14}H_3COOH$						$CH_3C^{14}OOH$					
	Carbon atom of hexose											
	1	2	3	4	5	6	1	2	3	4	5	6
Direct carboxylation	xx	x	—	—	x	xx	—	x	x	x	x	—
Glyoxylate cycle	xx	x	—	—	x	xx	—	—	x	x	—	—
Citric acid cycle	xx	xx	x	x	xx	xx	—	—	x	x	—	—

expected on the sole operation of the citric acid cycle but higher than expected on the sole operation of the glyoxylate cycle, the conclusion was drawn that both cycles must be operating. It is important to note that the magnitude of the effect under their conditions was small, which may explain the inability of Cutinelli *et al.* (272) to detect a similar effect in amino acids synthesized by *Escherichia coli* grown on labeled acetate and Ehrensvard *et al.* (278) working with *Torulopsis* or Gibbs (276) to observe this effect in glucose from starch obtained from autotrophically-grown *Chlorella* incubated with acetate-2-C^{14} in the light. The data observed with *E. coli* and *Chlorella* can be explained on the exclusive operation of a citric acid cycle.

Tartaric acid and itaconic acid.—Although the tartrates have been considered as metabolites for over 100 years, little was known of their metabolism until recently. A DPN-requiring tartaric acid dehydrogenase which catalyzes the oxidation of (−)-and meso-tartrate has been demonstrated by Stafford (279) in pea, bean, and wheat particulate and soluble fractions, and by Kun & García Hernández (280) in mitochondria from mammalian tissues. The apparent product of this oxidation, the keto or enol form of dihydroxyfumarate, has been reported in higher plants (281). In a continuation of this interesting series of papers, Stafford *et al.* (282) reported that roots, seeds, and leaves of higher plants contain an enzyme capable of oxidizing DPNH by diketosuccinate. The enzyme was called diketosuccinic acid reductase. Attempts to demonstrate the direct reduction of DPN by dihydroxyfumarate in these plant extracts were unsuccessful because of various complications. Kun (283) was able to demonstrate the oxidation of dihydroxyfumarate by DPN in animal mitochondria only when the reaction was coupled to a DPNH-oxidizing diaphorase system. The postulated reactions between tartrate and diketosuccinate are as follows:



46.

Further enzyme studies on the subsequent metabolism of the diketosuccinic acid were complicated by the spontaneous decarboxylation of the diketo-acid to hydroxymalonate (283). Extracts of a number of higher plants have been shown by Stafford (284) to contain a DPN-specific dehydrogenase which catalyzes the oxidation of the hydroxymalonic acid (tartronic acid) to ketomalonic acid (mesoxalic acid). The decarboxylation of ketomalonic acid would give rise to glyoxylic acid.

The biosynthesis of the tartrates in higher plants has received some recent attention. The low yield of radioactivity in tartaric acid isolated from grape leaves incubated with ascorbic acid-6-C^{14} [Loewus & Stafford (285)]

would appear to rule out a suggested relation between these compounds (286). The appearance of tracer in the internal carbons of the (+)-tartaric acid instead of the carboxyl carbons when grape leaves are exposed to $C^{14}O_2$ indicates that a sugar is the more likely precursor (287). Since carbons 2 and 3 of D-glucose and (+)-tartaric acid have the same configuration, Vickery & Palmer (288) have speculated on a possible relationship. It is important to note that no specific enzymatic reactions involving (+)-tartaric acid are known in higher plants. Strains of *Pseudomonas* have been reported to metabolize all three isomers of tartaric acid by means of inducible stereospecific dehydrases forming oxalacetic acid (289, 290). A similar dehydrase in tobacco leaves may be indicated by the interesting observation of Vickery (291) of the complete inhibition of citric acid formation in leaves cultured in tartaric acid at pH 2.8.

The synthesis of itaconic acid by the fungus, *Aspergillus terreus*, from glucose-1-C^{14}, acetate-1-C^{14}, succinic acid-1-C^{14}, and succinic acid-2-C^{14} has been reported in a series of papers by Bentley & Thiessen (292, 293, 294). They concluded that glucose was converted to itaconic acid by the following sequence:

$$\text{glucose} \xrightarrow[\text{citric acid cycle}]{\text{EMP}} \underset{\text{cis-aconitic acid}}{\begin{matrix} \text{CH—COOH} \\ \| \\ \text{C—COOH} \\ | \\ \text{CH—COOH} \end{matrix}} \rightarrow \underset{\text{itaconic acid}}{\begin{matrix} \text{CH}_2\text{—COOH} \\ | \\ \text{C—COOH} \\ \| \\ \text{CH}_2 \end{matrix}} + CO_2 \qquad 47.$$

The tracer labeling pattern of the itaconic acid is most readily explained by a removal of the carboxyl group attached to the primary carbon atom of the C^{14}-aconitic acid.

Ethylene.—The release of minute amounts of this unsaturated hydrocarbon by the fruits and flowers of higher plants has been known for some 25 years (295). Recently, ethylene has been detected during the vegetative growth of *Penicillum digitatum* (296). The ethylene-mercury complex of Young *et al.* (297) containing C^{14} when mixed 15:85 (by volume) with ethylene glycol was found to provide a satisfactory counting mixture by Spencer (298). It is interesting to speculate that ethylene could arise from ethyl alcohol by dehydration or from fumaric acid by decarboxylation. Obviously, the use of C^{14} offers advantages for determining the biosynthesis of ethylene, which is still unknown. Since ethylene is a symmetrical molecule, the powerful tool introduced by Wood (299) could prove useful.

Preparation of Various Compounds

Many papers have appeared during this period which are specifically concerned with the preparation of compounds discussed in this review. Methods for the preparation of D-xylose-3-phosphate (300), 2,3-diphosphoglyceric acid (301), D-erythrose-2-phosphate (302), and sedoheptulose di-

phosphate (303) have appeared. Srere *et al.* (304) have assembled the elegant techniques used in Racker's laboratory for the preparation of the intermediates and enzymes of the pentose phosphate cycle. A new method using *Escherichia coli* has appeared for the preparation of glucose-3,4-C^{14} (305). The C^{14} was shown to be evenly distributed between carbon atoms 3 and 4 of the glucose (306). Methods for the biosynthesis of sedoheptulose (307), fructose and glucose (308), and arabinose, xylose and galactose (309) uniformly labeled with C^{14} have also appeared.

Reviews

Some general treatments other than those quoted have appeared which are of considerable interest. Summaries concerned with the relation between the pentose phosphate and EMP pathways have been published by Bonsignore *et al.* (310), Szafranski (311), and Lioret (312). Janes (313) has reviewed the various respiratory mechanisms of higher plants in relation to the carbon pathways as well as electron transport. The mechanism, energetics, and properties of the individual enzymes of the citric acid cycle have been considered by Ochoa (314), while Wiame (315) has discussed its role in biosynthesis. An excellent summary on the chemistry and function of lipoic acid has been prepared by Reed (316). *The Carbohydrates* edited by Pigman (317) has appeared. This book is a complete revision and expansion of the *Chemistry of Carbohydrates* by Pigman & Goepp and includes, in addition to a thorough coverage of the nature and properties of carbohydrates, a chapter on photosynthesis and metabolism by Noggle.

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THE CHEMICAL REGULATION OF GROWTH (SOME SUBSTANCES AND EXTRACTS WHICH INDUCE GROWTH AND MORPHOGENESIS)[1,2]

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The first task is to define the subjects to be covered in this review and to relate them to other areas of knowledge that have recently been reviewed and to the broad trends of work on growth-regulating compounds.

It is now generally conceded that substances or extracts, which are not themselves nutrients in the accepted sense, profoundly affect the kind and quantity of growth. However, to classify these responses and to arrive at rigidly prescribed topics for this review is more difficult. Particular tests and test objects direct attention to different facets of the growth process, but these are not as distinct as much recent terminology may indicate. Therefore, certain general ideas on growth and development, with special reference to growth-promoting substances, should first be expressed.

The Chemical Induction of Growth in Relation to Morphogenesis

Stimuli from pollination, fertilization, and in early embryogenesis.—Pollination in the angiosperm prepares the way for the act of fertilization. Perhaps the greatest of all the stimuli to growth is fertilization for it produces from the unfertilized egg, which usually cannot grow, the zygote which in its development unfolds the complete characteristics of the organism. However, apomictic development of "embryos" occurs so that the growth stimulus may be communicated without the fusion of gametes. Apomictic development may occur nonrecurrently, if the egg (parthenogenesis) or the synergids are stimulated to form haploid embryos, or recurrently, if cells of the parent sporophyte (e.g., of the nucellus) are stimulated to form diploid embryos directly. There is a rare case (35) in which male nuclei, liberated into the embryo sac by the pollen tube, form "androgenic" embryos if the egg and synergids have been previously destroyed by artificial means. It is difficult to specify the stimuli (whether from nucellus, embryo sac, or pollen) which act upon the formative cells in these different cases of apomictic development, and how the cells which respond in these ways differ from other cells of the plant body. These questions obviously require answers in chemical terms.

Again, in the reproduction of angiosperms, the development of endosperm usually precedes the growth of the embryo; and it may persist vari-

[1] The survey of literature pertaining to this review was completed December, 1958.

[2] The following abbreviations will be used: 2,4-D (2,4-dichlorophenoxyacetic acid); IAA (indole-3-acetic acid); IAN (indoleacetonitrile).

ously in the development of ovules, as liquid, as cellular tissue, and even as storage tissue in some mature (albuminous) seeds. The liquid endosperm has been recognized as a potent source of stimuli which affect growth and morphogenesis in the immature embryo (120). However, the responsible growth-promoting substances may accumulate precociously in the endosperm in advance of the embryo's development. When this occurs (see example described below) the isolated fluids will induce growth in some normal mature cells. Even the act of pollination alone may exert stimuli distinct from those that operate after fertilization, for it may first stimulate the growth of the ovary, as Fitting (29) found in 1910 in the orchid. The stimulus of pollination to the growth of the ovary may be replaceable by a pollen extract, foreign pollen, dead pollen, or even by spores from quite different plants (e.g. lycopodium spores on *Cucurbita* stigmas).

However, the zygote or embryo is a center of growth as well as a focal point from which stimuli emerge to cause the development of the ovary wall or pericarp, as is evident in the formation of certain fleshy fruits. In this latter effect the embryo may often be replaced by other endogenous or exogenous stimuli; therefore, the chemical basis of these phenomena requires interpretation.

Other growth-regulating effects.—The twin aspects of growth—cell multiplication and cell enlargement—are commonly treated separately, as though they were subject to distinct stimuli and controlling factors. However, cells divide first and then they enlarge. These two growth processes frequently merge smoothly, the one into the other, and indeed they often overlap. Frequently enlarging or highly vacuolated cells divide, for cell division is by no means as confined to organized growing regions as is often supposed. Whereas cell enlargement has long been regarded as the sphere of influence of the "auxins," the more recently emphasized stimuli to cell division have been termed "kinins" (61).

A range of growth regulatory or morphogenetic stimuli operates in such phenomena as tuberization and the formation of other organs of perennation (often under such environmental control as the periodicity of light or temperature). In the physiology of cambial development, dormancy of buds, and abscisson of leaves and fruits, it is clear that the seasonal cycles of growth must be evoked ultimately by chemical stimuli. In the abnormal growth of tumors, whether incited by factors inherent in the genetic constitution of the host (48, 50) or by active agents such as bacteria (11) or viruses (7, 8), the ultimate stimulus to neoplastic growth surely must be chemical in nature.

Lastly, the transition of the growing point from the vegetative to the reproductive phase may involve chemical regulatory control. If, in this sequence of quite distinct morphogenetic events, the chemical stimuli are of a formative nature, many substances rather than a single flowering hormone would seem to be required. If, on the contrary, the stimuli merely release the growth of latent centers in the growing point, in which potentialities to flower are inherent, then a single chemical signal may suffice.

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Growth-promoting agents: terminology.—Very few chemical substances have hitherto been invoked as the stimuli which elicit this wide range of morphogenetic responses in angiosperms. For a long time the one known category of such stimuli was the auxins, dominated by the only natural, definitely known auxin, indole-3-acetic acid. This review will not dwell on the auxins per se; these have been reviewed frequently and recently (5, 129). In one or another of their forms, the coleoptile tests and the pea test focus attention on the principal effect of auxins which is to promote cell enlargement, so that some writers in this field use the term "growth" as though it were synonymous with "cell enlargement."

With the advent of one-directional chromatographic procedures, the ability of an extract to promote or suppress the growth of the oat coleoptile could be located in specific bands on paper strips. This approach has produced a plethora of "skyscraper diagrams," in which zones of the chromatogram are associated with growth-promoting or growth-inhibitory activity. When first done, as for example by Bennet-Clark *et al.* (4) and Luckwill (55), and as reviewed by Gordon (36), this was a major advance and indicated that the ability of plant extracts to promote extension growth was not confined to a single substance. However, experience with two-dimensional paper chromatography in other areas of work has shown the many errors that can be made in the facile identification of compounds by their position on such chromatograms alone (110, 112). When the separations are unidirectional, as in the auxin work, the resolving power of the method is very much lower, It is, therefore, no longer particularly instructive to multiply examples of these alleged growth-promoting "substances," which are designated solely by areas on the chromatograms, or even to indicate the multiplicty of inhibitors, unless the responsible compounds can be isolated and identified.

Comparative newcomers to the field of growth-regulating substances are the so-called gibberellins or gibberellic acids. These substances elicit, in essence, a particular kind of auxin effect; most dramatically, they release growth from the restrictions which operate in normally dwarf plants. These compounds and their effects have been subjected to recent and comprehensive review (113, 115) and, therefore, they will not be discussed in detail in this paper.

Although they are unrelated chemically to known "auxins," the gibberellins produce some similar effects. When gibberellins were first detected as products of the fungus *Gibberella fujikuroi*, it was not expected that they would subsequently appear as products of the higher plant. However, claims are now made for "gibberellins" in higher plants (74) and for their possible role in the breaking of the rest period, especially of normally biennial plants (50a).

There is some confusion between the detection of a response in the test object and the detection of a substance in a plant extract, however. Numerous papers (54, 69, 74, 76, 77) have been written to demonstrate the existence of gibberellins or gibberellin-like substances in a variety of plant

extracts. But the mere detection of a response in a test object is insufficient evidence that the substances that evoke it are the same as others known to be physiologically active. Indeed, the practice of identifying gibberellins or auxins solely on the basis of responses produced by extracts or solutions which are similar to those elicited by the pure substances should be discontinued, pending the isolation and purification of the substances in question. As surprising as it may seem, however, West & Murashige (130) and MacMillan & Suter (57) recently have claimed the isolation of substances from seeds of bean that appear to be chemically similar to, if not identical with one of the substances which was responsible for the original so-called "gibberellin effect," caused by the fungus *Gibberella fujikuroi*.

The stimuli to growth by cell division, whether communicated by synthetic substances, by artifacts produced from natural compounds, or by extracts of plants, have been attributed by Skoog & co-workers (61, 96) to substances which behave like kinetin (6-furfurylamino purine) and belong to a general class of so-called "kinins." There can be no question that many substances and extracts cause rapid cell multiplication, and the causal substances need to be specified. However, obvious problems are created if one regards one aspect of growth (cell enlargement) to be promoted wholly or predominantly by the class of substances known as "auxins" and another aspect of growth (cell division) to be stimulated by another class of substances to be known as the "kinins." These names alone do not lead to understanding; on the contrary, they may lead to confusion. A simple example will illustrate the problem.

A variety of synthetic substances, including 2,4-dichlorophenoxyacetic acid (2,4-D), are commonly regarded as growth regulators which operate in lieu of the natural auxin. Coconut milk may be regarded as a natural fluid that contains "kinins" because of its effect on carrot phloem explants, although the constituents of the coconut milk may not be normal for the carrot cells as they occur in the plant body! Neither 2,4-D nor coconut milk alone will induce maximum growth in potato tuber tissue (97). However, a suitable combination of coconut milk and any one of a great variety of 2,4-D-like compounds is effective if both members of a synergistic mixture are continuously present in the medium; then continued growth ensues by both cell division and cell enlargement (106). Is 2,4-D a "kinin" or an "auxin" toward the potato cells since the induction of growth does not occur in its absence? Because the coconut milk alone does not stimulate potato tissue to cell division, can it, or any constituent of it in the absence of the 2,4-D, be called a "kinin"?

Regardless of what they are called, coconut milk and 2,4-D both play a part in situations that induce growth in otherwise nongrowing cells. In the full understanding of their effects there should be the basis of an explanation of the kind of message that induces growth in many situations *in vivo*.

Therefore, the terms "auxin," "gibberellin," "kinin" need to be used with greater care; they may have little general significance when applied

beyond the limits of a particular test situation. Moreover, these terms should only be used in the full recognition that similar physiological responses in the test objects may result from very different compounds from those with which the response was first associated. This is indeed a major dilemma, for there is often no other obvious similiarity between such chemical agents than the ability to produce the response in question!

Virtually all the original rules which specify the chemical constitution of an auxin are now discarded (124). Within a group of closely related aryl-oxy acids certain relations between chemical structure and physiological action exist. Indeed, within a given group of auxin-like compounds their specificity may be strikingly related to known details of their chemical structure (89, 108, 127). However, the molecular characteristics of an auxin cannot be simply stated. Such a statement must embrace molecules as diverse as ethylene and certain straight chain compounds on the one hand, the familiar indole compounds on the other, and now even such highly complicated molecules as the gibberellins. One may here adopt a not unfamilar genetic analogy. Geneticists may be able to recognize that there are factors which determine different varieties and breeds of cats, even though they may be quite nonplussed by the factors which determine "catness." Thus, since the fundamental knowledge of how these growth-regulating substances really act is lacking and the precise site in the cell at which they operate is unknown, the descriptive terminology has become loose and confused. At present, it is far easier and more fashionable to multiply observations of growth-regulating effects than to isolate and identify the causal substances and determine how they work. In this review, therefore, a great deal of valuable but purely observational work is not mentioned.

Particular attention will be focused on the growth-regulating effects and compounds which are to be found most abundantly in solid or liquid endosperm, and on effects which are linked together because they occur in morphological situations in which immature embryos are normally nourished. Thus the growth-regulating factors in question arise from the most potent, natural situations of all, in which the egg becomes an embryo and then embarks on its development.

In retrospect, the preoccupation of plant physiologists with auxins, and especially with indoleacetic acid as a particular substance which is concerned mainly with a rather final aspect of cell growth (i.e., cell enlargement), may have diverted attention from many other types of natural growth factors and regulators. This is now being corrected by the attempted isolation and characterization of other growth substances and by the description of their effects. It is with these phases of research, now well launched, that this review is primarily concerned.

Sources of growth-promoting extracts.—It is very necessary to understand the morphological situations from which these growth-promoting effects originate. To illustrate this, reference will be made to the coconut, the caryopsis of maize, the parthenocarpic fruit of the banana, the fruit of horse-

chestnut, the female gametophyte of *Ginkgo*, a gymnosperm, and certain abnormal tumorous situations.

Test systems.—The principal test system consists of explanted pieces of carrot root from the secondary phloem, removed aseptically at such a distance (1 to 2 mm.) from the cambium that the explant contains no cambium and is composed of cells which have ceased to divide in the intact root, though they sluggishly expand when isolated. The dramatic ability of the coconut milk, or equivalent fluid, to incite such explants to proliferate is still the basis of a very useful assay system. If the tissue culture is one which grows around an explant, the cultured tissue remains irregular and undifferentiated and root formation is virtually absent. The measure of growth in such a system may be by the weight increment or the cell number determined after maceration. It may also be expressed in terms of average cell size based on these measurements. The carrot assay system is generally applicable because explants from a great variety of carrots respond quite dramatically to applications of coconut milk, although different varieties from different sources and even different roots of the same variety furnish explants which differ in the degree of growth so stimulated (105).

The carrot assay system is more useful than some others because it lends itself to standardization and to quantitative work, and the material is generally available. It is much more convenient than an assay based on immature embryos. It also seems more generally applicable than the tobacco pith method which depends on internal divisions of giant callus cells of pith, although this method has led to important developments in the hands of Skoog and his collaborators (60, 61).

Morphology and the Sources of Growth-inducing Substances and Extracts

Coconut milk or the liquid endosperm of Cocos nucifera.—The morphological nature of this source of growth-promoting material is now familiar. At maturity of the fruit, the embryo, embedded in the solid endosperm beneath the micropyle, is very immature and undifferentiated. At this stage the nut contains a cellular tissue which had, itself, grown within the cavity of the nut, no doubt at the expense of nutritive materials contained in the coconut milk (or coconut water as it is sometimes called). The coconut milk is a liquid endosperm which contains some free cells and free nuclei. When the embryo eventually grows, it sends out a part of its cotyledon into the central cavity, where it forms a spongy, cellular tissue that grows at the expense of the nutriment contained in the endosperm. Thus, the food material for the embryo and the stimuli which promote the cell growth in the cotyledon are laid down, precociously, in advance of the development of the coconut embryo. The ability of the coconut milk to promote cell divisions in carrot explants, under the conditions of the carrot assay system, is not restricted to a limited phase of development of the fruit but is spread over a period of time after pollination (99). Whether the endosperm is

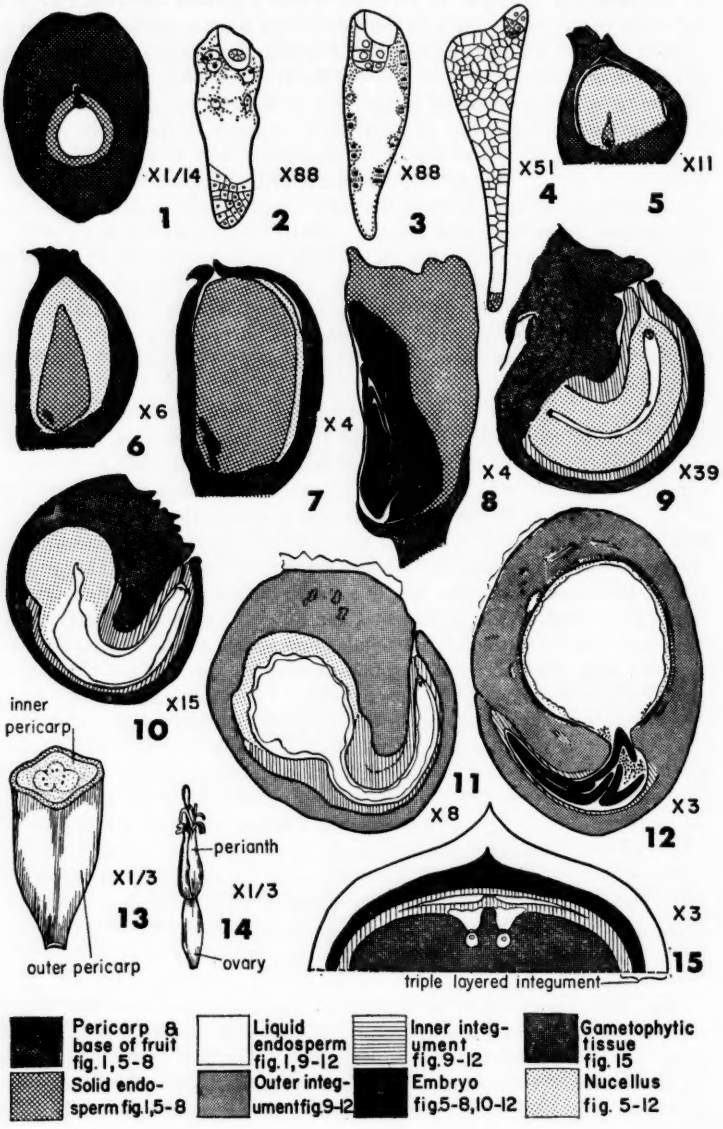
entirely or only partly liquid, its activity persists in the mature nut until the cotyledon is enlarged. In fact, the activity persists somewhat even in the tissue of the young embryo (99). Although some workers have used an infusion of the solid endosperm (58), all indications are that the activity of this tissue is much less than that of the liquid endosperm.

The immature caryopsis of maize (corn in the milk stage).—The first successful attempt to produce an equivalent response in the carrot explants by the use of a plant extract other than coconut milk was achieved with a warm water extract of lyophilized immature fruits of corn (*Zea mays*) (15). The growth-promoting ability of the corn fruit differs from that of the coconut because it has reached maximum activity two weeks following pollination, after which it rapidly disappears from the mature grain. In the coconut, the activity is present in the fully formed nut (99). It is pertinent, therefore, to describe the condition of a corn fruit at this stage and to indicate the mode of development of the most important structures that may furnish the activity in the extracts. The following account is based on the work of Randolph (78).

In the development of the corn fruit, the embryo sac enlarges early to form a liquid-filled cavity (Figs. 2 and 3). Within this embryo sac, the egg and synergids lie at the micropylar end and the antipodal cells at the other. For a brief period (up to three days), numerous endosperm nuclei exist free in the embryo sac. Embedded within the nucellus, the large embryo sac soon becomes filled with a cellular tissue as endosperm nuclei multiply by mitotic division and walls are formed between them (Fig. 4) This growth within the embryo sac is like that of a tissue culture growing independently within the nucellus, which furnishes the nutritive material. As shown by Randolph, the formation of the cellular endosperm within the embryo sac proceeds from the micropylar region and extends towards the chalaza. In corn, the true homolog of the coconut milk is the embryo sac fluid by which the eventual cellular content of the embryo sac is nourished during its growth while the zygote is still quiescent (cf. Figs. 1 and 3). It is doubtful whether this fluid has yet been strictly assayed because, in the period up to two weeks of development after pollination, the embryo and the fruit advance considerably and attain a condition shown in Figures 6 and 7. At this point almost all traces of the embryo sac liquid have disappeared, and the now well-organized embryo is being nourished by a bulky endosperm composed of cells which are rich in soluble material. Thus, much of the growth-promoting activity, which is obtained by extracting whole but not fully mature fruits, may well be more analogous to that which might be obtained from the more solid endosperm tissue of the nut, in the case of the coconut (cf. Fig. 1).

The immature fruits of horse-chestnut (Aesculus woerlitzensis).—For a long time, a parallel situation to those of corn and coconut was not found among the dicotyledons. However, in immature fruits of the walnut, similar activity in the carrot test was discovered (99). In this plant the cotyledons grow down into a sap-filled cavity. Later the activity was found in the young

MORPHOLOGY OF THE SOURCES OF GROWTH FACTORS



fruits of *Aesculus* (85, 106). When harvested in early August, the *Aesculus* fruit contains a long, curved embryo sac which has grown from the stage in which it first appears (Fig. 9) to the more enlarged structure which is shown in Figures 11 and 12. Early in its development and before the nucellus has disappeared, the embryo sac contains the egg apparatus towards the micropyle and small antipodal cells at the chalazal end. Scattered around this swollen embryo sac are many free nuclei (Fig. 9). Between mid-July and mid-August (Fig. 12), the fluid is even more abundant and, surprisingly, contains a large gas bubble. When pierced under suction, the liquid contained in these embryo sacs can be withdrawn, and it is this opalescent fluid which has been termed "horse-chestnut milk." During the course of development, the nucellus virtually disappears (Fig. 12), and the embryo sac comes into direct contact with the inner integument. The outer integument becomes thick and fleshy, and the separation between the two integuments becomes indistinct. Extracts of the solid residual tissue of the fruit, therefore, are derived mainly from the integuments which are now massive and contain much starch. A minute amount of the peripheral, free nuclear, or at this stage cellular, endosperm must also be included in the solid residue.

As the embryo increases in size, the outer integument becomes thin, leathery, and brown from tannins. During development, therefore, the integuments represent a storage organ and furnish nutrients to the developing embryo and endosperm. They replace the nucellus which is absorbed early in the growing season. It is not surprising, then, that the growth-promoting

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MORPHOLOGY OF THE SOURCES OF GROWTH FACTORS—
A DIAGRAMMATIC REPRESENTATION

FIG. 1. Fruit of *Cocos nucifera*, showing fibrous mesocarp, stony endocarp, solid and liquid endosperm with embryo (longitudinal section).

FIGS. 2–8. *Zea mays;* Figs. 2–4 (after Randolph, 1936), stages of the embryo sac 34, 72 and 96 hours after fertilization. Note the liquid content and antipodal cells in Fig. 2, nuclear endosperm in Fig. 3, the cellular endosperm which develops first near the embryo in Fig. 4. Figs. 5–8 show the progressive development of endosperm at the expense of the nucellus and of the embryo at the expense of the endosperm respectively; Figs. 5, 6, and 7 at 4, 10, 18 days, respectively, from fertilization; Fig. 8 mature grains in longitudinal section.

FIGS. 9–12. The ovule of *Aesculus*, showing the progressive enlargement of the liquid-filled embryo sac at the expense of the nucellus and the development of the surrounding integument and tissue of funiculus.

FIG. 13. Part of parthenocarpic fruit of "Gros Michel" banana, showing the inner fleshy and the outer fibrous pericarp.

FIG. 14. The female flower of banana at receptivity.

FIG. 15. Median section through ovule of *Ginkgo biloba*, showing a triple layered integument which encloses the female gametophyte and 2 archegonia with large egg cells which develop into a free cellular proembryo [after Coulter & Chamberlain (16a)].

(FIGS. 1 to 15 prepared with the help of Dr. H. Y. Mohan Ram and Mr. A. List).

materials which are contained in the sap of the *Aesculus* embryo sac (occupying almost the entire vesicle) and those of the remaining, more solid tissue (which includes a little peripheral endosperm as well as the integuments) have a very different chemical nature (see pp. 396).

The female gametophyte of Ginkgo.—A very different morphological situation, but one with nutritional parallels, has also furnished growth-promoting extracts that induce growth in carrot cells; this is the female gametophyte of *Ginkgo* (Fig. 15). In this case, the activity resides in a haploid tissue (also termed endosperm by morphologists), which is formed prior to fertilization. The tissue resembles the angiospermous endosperm because it is a nutritive tissue for the development of the archegonium and the embryo. In view of the activity of an extract of *Ginkgo* endosperm toward carrot tissue (99) in which it replaces coconut milk, it is interesting that Tulecke (119) has made the pollen of *Ginkgo* proliferate and grow by the use of coconut milk!

The developing parthenocarpic banana fruit.—This material is of interest because, in nonseeded banana plants, bisexual flowers have vestigial ovules in their carpels. Lacking the stimulus of pollination and that which would normally emanate from the seed, however, the fleshy pericarp grows to form the soft edible tissue of the fruit. An extract made from the innermost layer of the pericarp of the young fruit (Fig. 13), which constitutes the main layer within which the development occurs, is also able to stimulate the growth of carrot cells (109). This situation is very different morphologically from the other examples which have been described, because the growth-promoting activity here resides in an immature fleshy pericarp of the fruit, whose development is incited neither by pollination nor by the presence of viable embryos but by a genetically determined feature of the plant.

Imbalance in growth regulators in relation to normal and abnormal growth: tumors.—Parenchymatous cells in mature plant tissues, and sometimes even buds of trees, may cease to grow in spite of being ideally provided with organic and inorganic nutrients. Tests of certain mature storage tissues (98) and of dormant buds of a tree (98) indicate that inability to grow may be caused by inhibitory substances. These inhibitors prevent response to stimuli, such as those contained in coconut milk, even when the latter are furnished exogenously. Thus, the growth of such parenchyma cells may be controlled by the interaction of factors that promote cell division with inhibitors that tend to suppress it (98, 99, 100). In normal development, a nicely balanced situation prevents the runaway growth characteristic of callus tissue. At least to a limited extent, such tissues often show renewed growth in the healing of wounds. However, when the hormonal balance is disturbed by an excessive supply of the stimuli which cause cell division, rapid proliferative growth ensues. This is familiar in tumors of crown gall, incited by the crown gall organism [cf., Braun (11)], in many insect galls, and in virus tumors such as those studied by Black (7). In such cases it is reasonable to assume that, for one reason or another, the cells have re-

ceived stimuli toward cell division and the "daughter" cells may remain autotrophic in this respect. It is, therefore, significant that extracts of certain tumors have stimulated carrot explants to grow (100) and that the tumorous cells resemble the carrot cells under the stimulus of coconut milk, because both contain a protein moiety which is richer in hydroxyproline than the cells of the normal tissue from which they were derived (111).

Stimuli to orderly and disorderly growth: growth of free cells.—Stimulated by the different extracts mentioned, the growth of carrot explants has invariably been unorganized. Recently (102) the growth of dissociated carrot cells, freely suspended in liquid, has been studied in the presence of coconut milk. The free carrot cells respond to the coconut milk stimulus in a variety of ways, but they finally reach a fairly uniform multicellular condition. The growth is entirely random and unorganized, for each cell grows independently when unrestricted by its surroundings. The resultant cell forms and patterns have been described.

Before orderly growth is resumed, a new type of stimulus is needed. This stimulus arises from within the cellular mass when some of the cultured cells lose their contents, lignify, and differentiate to form the tracheid-like elements which have so often been observed in tissue cultures. However, stimuli radiate from this center of differentiating cells, causing cells to divide in an orderly fashion (101). By divisions tangential to this radial stimulus, a cambial or pericycle-like mass is formed, comprising a sheath of dividing cells that encloses a sphere of cellular tissue. As a consequence of the coconut milk without and the stimulus from the differentiating cells within, the contained nodule or spherical mass of tissue now grows in an orderly manner.

Orderly development takes place first by the initiation of a root tip which can occur in a liquid medium while the tissues are rotated around a horizontal axis and are not, therefore, geotropically influenced. Later, however, the nodule or nest of cells with its root initial will form a shoot at the opposite end of the axis; such development requires exposure to air and orientation to gravity. Thus a small, embryo-like structure is developed from originally differentiated but dissociated cells, in the presence of growth-promoting substances that normally nourish growing embryos. From these structures, carrot plants with dissected leaves and a secondarily thickened root have been developed in aseptic culture by Steward, Mapes & Mears (101).

Growth induction and protein synthesis.—A special feature of the growth-induction phenomenon due to coconut milk involves protein synthesis. Not only is protein synthesized in the stimulated cells, but a particular kind of protein, rich in hydroxyproline, is formed. The proline is directly incorporated into the protein and is later hydroxylated to give a hydroxyproline-rich protein moiety which is structural in character, for it is not metabolized by the growing cells [Steward & Pollard (103)]. Since this type of protein seems to occur in other rapidly proliferating cells (111), it may prove to be characteristic of cells in this state.

Biochemistry and morphogenesis.—A range of systems in which biochemistry in relation to morphogenesis may be investigated is now available. In other types of work Skoog *et al.* (96) have made progress in the investigation of the control of organ formation by specific substances applied under tissue culture conditions.

In the morphological situations illustrated (Figs. 1 to 15), there is still greater scope for the detection, isolation, and assay of growth-regulatory compounds with specific functions than has been exploited fully. Interest in the growth-regulatory substances extends beyond their identity to the means by which their action is coordinated, controlled, or even suppressed in normal development. With this in mind, present knowledge of the effective substances which are present in each of the sources of growth-regulating or formative substances illustrated in Figures 1 to 15 will be summarized. Large scale and extremely detailed fractionation of these materials is needed in order to obtain a sufficient quantity of the critically pure substances for the determination of their chemical constitution.

The Fractionation of Extracts Which Induce Growth

Responses due to coconut milk.—The use of coconut milk as a growth-promoting supplement is derived from work of van Overbeek *et al.* (121, 122) in the culture of excised immature *Datura* embryos, which failed to grow unless the basal medium was fortified with unautoclaved coconut milk. The fraction of the coconut milk promoting organized growth was heat-labile, whereas the fraction promoting unorganized callus growth of the embryos was heat-stable. Cutter & Wilson (17) later found similar effects of coconut milk on the growth of the coconut.

From later work on the growth of free cells of carrot, the distinction between the so-called "embryo factor" and the "callus growth factor" may disappear, for autoclaved coconut milk may promote either disorganized or organized (embryo-like) growth, according to the state of the material which undergoes the response (101). Ball (1) observed that stem tips of *Lupinus* grew and developed into whole plantlets when unautoclaved coconut milk was added to the medium; but pieces subjacent to the tips, in the same medium, proliferated in an unorganized manner. Again, the difference in the degree of organization of the new growth was more dependent upon the material than upon whether or not the coconut milk was autoclaved.

Using a reproducible technique for the controlled growth of plant tissue cultures in a liquid medium (16), Caplin & Steward found that tiny explants of secondary phloem from carrot root grew into an undifferentiated mass at many times the basal rate of growth if White's liquid medium was supplemented with 15 per cent by volume of whole coconut milk before it was autoclaved (15). The stimulus of the coconut milk could not be replaced by a number of commonly used nutrient extracts or plant-growth stimulators. The discrepancy between growth in the basal and supplemented media is really greater than is shown by the data of fresh weight alone; this is because

the increment of weight which occurs in the White's basal medium is predominantly cell enlargement, while the coconut milk specifically promotes cell division.

Many others have found coconut milk to be a stimulant which induces and maintains growth in a variety of plant tissue cultures. Duhamet found that the tissue cultures from Jerusalem artichoke, tobacco, salsify, carrot, grapevine, and *Parthenocissus* were all stimulated far more by coconut milk than by indoleacetic acid (21 to 26). Gautheret (33) observed that coconut milk could be used to initiate and maintain growth in tissue cultures from a number of sources. In some cases where tissues responded to IAA, coconut milk was actually inhibitory, probably because, in the combination, the effective auxin concentration may have reached toxic levels. Using tissue from the Jerusalem artichoke, Duhamet & Gautheret (27) verified the fact that both coconut milk and IAA stimulate growth but coconut milk produces a more abundant and less differentiated type of growth than does IAA. Henderson *et al.* (43) found a striking effect of coconut milk on sunflower callus tissue, while a similar effect was observed by Nickell (67) on the growth of virus-induced tumor tissue of *Rumex*. By the addition of coconut milk (together with 2,4-D) to their medium, Morel & Wetmore (62) obtained the first tissue culture from a monocotyledon. Tulecke (119) also found coconut milk promoted growth of a tissue culture obtained from *Ginkgo* pollen. Studies have been made of the effect of coconut milk media on the carbohydrate and protein composition of tissue cultures of carrots [Goris (37)] and of salsify [Lioret (53)].

These diverse results show beyond all doubt that the contents of coconut milk induce a remarkable degree of growth by cell division, which cannot be reproduced by mixtures of known plant nutrients and hormones. The attempt to isolate and identify the responsible factors, however, has proved to be a very complex task.

Fractionation of coconut milk.—The first attempted isolation of active components of coconut milk was made by Van Overbeek, Siu & Haagen-Smit (123), who found the "embryo-factor activity" to be considerably concentrated in an 80 per cent ethanol extract of a lead acetate precipitate, after the removal of an inhibitory fraction. Probably because of the complex assay system used, this work did not lead to the isolation of any distinctive component of the coconut milk.

Summarizing their own work, Steward & Shantz (105) showed that relatively crude fractions, obtained by serial precipitation from whole coconut milk concentrate by the use of increasing concentrations of ethanol, showed partial activity in all fractions and complete activity only when the separated fractions were recombined. De Ropp *et al.* (20) reported that passage of coconut milk through an activated charcoal column yielded one per cent of adsorbed solids which could be eluted with a chloroform-methanol mixture. The eluate induced some growth of carrot root tissue, which was considerably more than that caused by IAA alone; but the response only

approached that of whole coconut milk when the filtrate and eluate from the column were recombined.

De Ropp (19), following Haagen-Smit & Siu, suggested that the coconut milk effect might be due to a favorable balance of already known nutrients and growth stimulators. Paris & Duhamet (72) and Paris (71) claimed that a mixture of known vitamins and amino acids stimulated the growth of a number of tissues in a manner similar to coconut milk, although to a lesser extent. From these results, Paris & Duhamet also felt that the activity of coconut milk arose from a mixture of known substances. The experiments of Paris & Duhamet (72) were not confirmed by Shantz (84); rather, the earlier results of this laboratory were substantiated. One may now dismiss all these negative claims by the assertion that it is no solution to deny the needle in the haystack because one can only see the hay!

Using a procedure applied to several hundred gallons of coconut milk and involving precipitation by mercuric acetate as the initial step, Shantz & Steward obtained a partially active concentrate from whole coconut milk, from which three active crystalline compounds were eventually isolated in small amounts (88). These substances, one of which was later identified as 1,3-diphenylurea (90), were active only when added to the culture medium in combination with casein hydrolysate. The kind of evidence for the activity of these compounds toward carrot tissue is shown by many experiments with diphenylurea (105). The effects of these isolated compounds were much more dependent upon inherent and variable characteristics of the individual roots from which the tissue explants were removed than is the effect of whole coconut milk.

Mauney *et al.* (58) used a similar precipitation procedure to prepare an active concentrate from an aqueous extract of the more solid endosperm of the coconut. No pure substances were isolated, however, and despite the title of a paper which claimed the "purification and properties of a growth factor from coconut," the activity was only concentrated by their procedures. Chromatographic analyses showed the concentrate to be an exceedingly heterogeneous mixture.

More recently, Shantz, Pollard & Steward (87) have again fractionated whole coconut milk, using ion exchange columns in the initial stages. Although the operations summarized (87) were complex, approximately 95 per cent of the initial dry weight passed through both anionic and cationic resins to emerge as a "neutral" fraction from which all ions had been removed. The material held by the resins consisted mainly of inorganic ions, amino acids, and a highly "active" fraction which appeared to he held more by adsorption than by salt formation. This fraction gave a positive, although somewhat variable, growth response at very low concentrations (on the order of 50 p.p.m.), while the "neutral" fraction was completely inactive. Activity comparable to that of whole coconut milk was obtained only when the "active" and "neutral" fractions were recombined and tested in the presence of an amino acid mixture, such as casein hydrolysate.

Therefore, it is now well established that coconut milk acts as a potent stimulus to the growth of some plant embryos and to many cultures of parenchymatous tissues. A comparable rate of cell proliferation is not obtainable by the use of known nutrients or growth regulators in the culture medium. The coconut milk response, however, results from the interaction of a number of substances, which fall into three distinct categories: (*a*) the free amino acids (as reduced nitrogen compounds), which are replaceable by casein hydrolysate, by a synthetic mixture, or even by urea (93); (*b*) a substance or substances in a "neutral" fraction which is not adsorbed by ionic resins or by charcoal, is itself unable to induce growth, but is highly synergistic with the "active fraction" mentioned below (87); and (*c*) the "active" fraction, comprised of the growth-promoting substances proper which are effective in low concentrations (on the order of 1 p.p.m.) and are characterized by strong ultraviolet absorption. These substances are strongly held by ion exchange resins or charcoal, and together they comprise a complex mixture of active growth-promoting substances (87). At least three of these active substances have been isolated in the form of recrystallized substances (88), but the presence of still other active materials in the extracts has been amply demonstrated. None of the active substances yet isolated correspond to known purines or pyrmidines, nor can any of the naturally occurring purines or pyrmidines duplicate the effects of coconut milk; neither has the substance kinetin (61) or any similar substance yet been isolated from the coconut milk.

Effects observed with the immature caryopsis of Zea mays (*corn in the milk stage*).—The first effective natural substitute for coconut milk was found (15, 99) by extracting lyophilized, immature corn grains at the stage of their development shown in Figure 7. Prior to this, corn pollen and extracts of the developing fruit had figured in diverse ways as sources of growth-stimulating activity, principally with reference to the growth of the fruit [for bibliography see the paper of McLane & Murneek (59)]. As shown by references cited in this latter paper, the main substances held to be responsible for the growth-promoting activity of corn were IAA and a hypothetical substance termed syngamin, which supposedly originated after fertilization and was associated with the development of the embryo. In fact, in the culture of excised embryos of corn, Haagen-Smit, Siu & Wilson (39) found that an extract of the corn kernel was effective, whereas coconut milk was not.

Claims that developing corn grains contained the ethyl ester of IAA were first made (79) and then qualified by the idea (41) that the ester was an artifact of alcoholic extraction. Observations on the auxin activity of immature corn, as shown by the coleoptile test, have led to the view that most of the activity is not attributable to IAA or to ether-extractable acidic substances (28). In this respect, these later observations confirm the earlier ones of Haagen-Smit *et al.* (39). The earlier attempts to obtain growth-promoting activity from corn extracts, and the obvious parallel between coconut milk and corn in the "milk" stage, have prompted several attempts to

show activity of corn extracts. The phrase "corn milk" refers variously to a pressed sap from the immature grain or to an extract or infusion of the immature grain. In any event, it must include material from the more or less cellular tissue of the embryo sac and also from the surrounding nucellus, and, if obtained later in development, from the embryo itself (see Figs. 5–8). In four papers by Netien & co-workers (63 to 66) in which the test system was a carrot tissue culture, "corn milk" was said to be as active as coconut milk in promoting growth, but the active factors were distinct from IAA. They were also said to be not entirely heat-stable, and the ether extract contained part of the activity, whereas the residue apparently showed two active and one inhibitory subfractions (65). More recently, three groups of substances have been separated on ion exchange resins by Beauchesne (3); two of these groups stimulated cell division, and the third group stimulated cell elongation especially.

In a different investigation Györffy *et al.* (38) applied the isolation procedures of Shantz & Steward (88) to a "corn milk" preparation. The growth-promoting activity was found in the precipitate with mercuric acetate and was subsequently concentrated in a butanol extract. The conclusion was that the activity was due, in part, to amino acids and, in part, to other substances which act in a manner similar to the growth stimulation due to coconut milk. Against this background, the present position in this laboratory was summarized in recent presentations to the American Society of Plant Physiologists (92, 107) and a symposium summarized by Salisbury (80a).

Shantz & Steward (92) have summarized the large scale fractionation of an extract of immature corn grains as follows: "Adsorption on charcoal followed by elution with acetic acid gives a concentrate of high activity. Further fractionation by chromatography yielded several crystalline substances of a purine-like nature. Three of these have been positively identified as adenine, adenosine, and uridine. These crystalline compounds have slight activity when tested alone in the carrot assay test, but they may act as co-factors in the natural extract. One substance, small amounts of which have been isolated in the non-crystalline but chromatographically homogeneous state, has certain indole-like properties, and has features in common with glycosides in the infrared spectrum. This compound stimulates proliferation in tissue cultures at low concentrations; it induces rootlet formation on explants from some carrot roots, and also induces elongation in *Avena* coleoptiles." When the paper of which the above was an abstract was communicated, it was reported that hydrolysis of this compound gave indoleacetic acid and a sugar moiety which was chromatographically identical with arabinose.

Although this isolation from corn has led to what seems to be a distinctive growth-promoting substance, this does not mean that this substance is responsible for the entire growth-promoting activity of the original corn extract. On the contrary, all the evidence suggests that other substances yet to be isolated and purified contribute to this, at least in part. Therefore,

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the situation in corn resembles that of the coconut milk in that the entire activity is due to a complex of growth-promoting substances, which interact with the nitrogenous compounds that occur in casein hydrolysate. Doubtless, if the extracts could be made in sufficient quantity from more specific morphological regions, such as the embryo sac or the nucellus (see Figs. 2 to 8), the biochemical evidence might seem to be less complicated and more specific for each region.

Immature fruits of Aesculus woerlitzensis (*horse-chestnut*).—The fruit of horse-chestnut has also yielded activity in the stimulation of growth of carrot explants at a stage of development in which material is obtained alternatively as a liquid from the embryo sac and as an extract of solid cellular tissue, which consists primarily of the integument (see Fig. 12). Large collections of these fruits were fractionated. The liquid content of the embryo sac was worked up separately, principally by solvent extraction culminating in chromatography on paper in a butanol-acetic acid system. The solid residue, on the other hand, was first extracted with ethanol; the ethanol extracts were submitted to fractional precipitation and subsequent solvent /solvent partition in an automatic Craig-Post extractor. Thus, the growth-promoting activity of the *Aesculus* fruits has two morphological sources (the embryo sac and the integument) which, not unexpectedly, yield growth-promoting substances of two entirely different kinds. The active materials from these two regions are summarized below.

The liquid content of the embryo sac (*liquid endosperm*).—At least five definitive fractions were obtained (91). Each of these substances, when eluted from the appropriate regions of the paper, had characteristic ultraviolet absorption curves. When the paper was presented, these curves were shown, and Professor R. F. Dawson drew attention to the fact that one of these substances could, from its ultraviolet behavior, be chlorogenic acid. This proved to be so.

The liquid content of the embryo sac of the horse-chestnut thus yields several distinctive substances, each of which is separately active; and the overall growth-promoting activity of this material is due to a complex mixture of substances.

The integument of Aesculus.—The biochemical fractionation of the solid residual tissue of the *Aesculus* fruits led to an unexpected type of growth-promoting substance. Here the activity resides in certain phenolic compounds and especially in substances which are interpreted as typical leucoanthocyanins.

Leucoanthocyanin-like, growth-promoting materials in horse-chestnut and coconut.—Growth-promoting activity in the carrot assay, due to leucoanthocyanin-like materials, was first observed in extracts of *Aesculus* fruits (85), although later it was found by solvent-solvent fractionation of coconut milk which had been concentrated by evaporation.

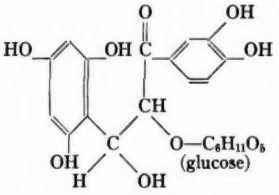
Shantz & Steward (85) described the fractionation of the integuments from immature fruits of the horse-chestnuts, as follows: "Fractionation of

these extracts has now shown that the activity is due to a complex range of substances. One active substance which occurs in large amounts in both the liquid and solid endosperm, has been isolated and shown to belong to the class of leucoanthocyanins. It occurs naturally as a monoglucoside, and when used at a concentration of about 20 p.p.m. in the medium, it shows growth-promoting ability approaching that of whole coconut milk at a concentration of 10 per cent. The aglucone is also present in the endosperm and has been isolated, but this has relatively little activity. The occurrence of the same or similar substances in coconut milk has been demonstrated."

It should be recognized that this is a particularly difficult class of natural substances to deal with [see Geismann & Hinreiner (34)]. These substances are extremely difficult to purify, have proved virtually impossible to crystallize, and [with one possible exception (49)] no substance belonging to this class has been wholly or even partially synthesized. Nevertheless, the leucoanthocyanins and related substances are so widely distributed in plants that any suggestion of their biological activity has important consequences.

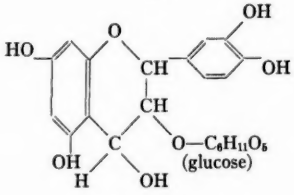
The corrected elemental analysis (106) of the isolated leucoanthocyanin-like material corresponded to C_{21} H_{29} O_{13} which agrees moderately well with the leucoanthocyanin formula A, C_{21} H_{24} O_{13} based on the formulation of Robinson & Robinson (80), or with the ring structure shown at B, which is based on A after ring closure (2, 117).

(A)



$C_{21}H_{24}O_{13}$

(B)



$C_{21}H_{24}O_{12}$

There can be no doubt now that many of the extracts which exhibit growth-promoting qualities toward carrot explants contain leucoanthocyanin and similar materials. This fact raises many interesting questions.

Significance of leucoanthocyanins.—Leucoanthocyanins are very widely distributed in plants. They occur in the by-products of wood, in many non-green parts of plants, such as petals of flowers, in saprophytic angiosperms, and in fruits, where they are often responsible for bitter flavors and astringency, especially prior to ripening. It may be more than coincidence that the young banana fruit is a rich source of leucoanthocyanins and also yields extracts which have growth-promoting properties. Work on *Eucalyptus* shows suggestively that there is much leucoanthocyanin in the cambial region at the time when this tissue is active [Hillis (44)]. It is an attractive hypothesis that the leucoanthocyanins represent a physiologically active

molecule concerned in some way with growth, whereas the anthocyanins are the inactivated storage product.

There seems no obvious point of contact between the structure of leucoanthocyanins and properties calculated to cause cell division. The catechol moiety of the molecule may furnish a link with oxidation effects; the effect of catechol itself on the respiration of potato disks [Boswell & Whiting (10)] may here be recalled, and especially the maintained stimulatory effect of a tissue extract (9) which could well have owed its properties to leucoanthocyanins. Surprisingly, however, there is some evidence which tends to link anthocyanin synthesis with nucleic acids (118); and this may relate the leucoanthocyanin effects on growth with nucleic acids and protein synthesis. Thimann & Radner (118) have shown that anthocyanin formation is inhibited by a number of purine analogues, including kinetin, and that this inhibition can be reversed by riboflavin (118a). Anthocyanin formation is also inhibited by copper-complexing reagents, and to a lesser extent by catechol (27a), while sugars, meso-inositol, and quinic acid probably act as intermediates in anthocyanin synthesis (117a). Therefore, a number of different observations suggest that there is some focal point in metabolism upon which phenolic compounds and leucoanthocyanins converge so that they interact with purines, nucleic acids, and protein synthesis in ways that affect cell division.

"Auxins" Other Than Indoleacetic Acid

The recent evidence indicates that many "auxin"-like substances other than IAA occur in plants. Often free IAA accounts for only a small part of the total activity detected in the *Avena* coleoptile or some similar test. This subject has been reviewed by Bentley (5). The many unidentified, but active, substances form zones on chromatograms which are associated with response in the test system. Some of these substances are indole derivatives and have either been isolated or more completely identified; these include the indoleacetonitrile (IAN) of Henbest *et al.* (41, 46), the indolepyruvic acid of Stowe & Thimann (114), and the indoleacetic acid ethyl ester of Redemann *et al.* (79).

The identified substances, however, may be only a fraction of the total number of the compounds active as auxins. How far the unidentified "auxins" might be active in other ways than are detectable by the usual auxin tests is not known (as, e.g., by tests which measure cell division specifically). Some *Aesculus* extracts which have been concentrated with respect to their ability to promote growth by cell division certainly had activity in the auxin tests (91). A list of representative recent papers shows the multiplicity of auxin-like natural growth factors which have been recorded. Such a list is as follows: Luckwill (56), at least four auxins in ether extracts of apple seed, none of which is IAA; Vlitos *et al.* (125, 126), an unidentified indole compound said to be the predominant auxin of Maryland Mammoth tobacco; Linser *et al.* (52), ten indole-like compounds in *Brassica* extracts,

including at least two active substances in addition to IAA and IAN, and an active nonindole compound; Schoen & Morel (81), two unidentified active substances in extracts of Jerusalem artichoke; Nitsch & Nitsch (70), six unidentified active substances in ether extracts of various tissues and tissue cultures; Braun & Naf (13), a nonauxin growth factor in crown gall extracts; Schwarz *et al.* (83), several unidentified auxins besides IAA, IAN, and indoleacetic acid ethyl ester in normal and tumor tissues; Kefford (47), an unidentified growth-promoter termed "accelerator α," in addition to IAA and IAN in extracts of cauliflower; Raadts & Söding (75), IAA and an unidentified weakly acidic growth-promoter which diffuses from *Avena* coleoptile tips; Housley & Bentley (45), IAA, IAN, and several unidentified growth promoters in the acid and neutral ether extracts of cabbage; Britton *et al.* (14), IAA, IAN, and an unidentified indole-type auxin and a nonindole auxin in tomato seedling roots; Fukui *et al.* (32), two unidentified growth-promoters in the neutral ether extracts of lettuce seedlings; Farrar *et al.* (28), at least two unidentified substances, not extractable with ether, which comprise most of the auxin activity in immature maize kernels; Hemberg (40), four active acidic substances and two active neutral substances in the ether extracts of maize kernels; and Fukui *et al.* (31), IAA and two other active acidic substances in the ether extract of corn (*Zea*) pollen, and a nonindole active substance in the neutral fraction.

These examples demonstrate the heterogeneity of the auxin-like substances from plants. Moreover, nearly all of the works cited deal only with ether extracts; the problem will be complicated even further when an equal amount of work is done on the substances which are not extracted by ether. However, as mentioned earlier, the repeated demonstration of activity in numerous zones on paper chromatograms can accomplish little more; the time has come to isolate and identify these many natural plant growth substances. In so doing, use should also be made of other tests which emphasize other aspects of growth such as cell division.

Synergistic Interactions with Plant Growth Substances in the Stimulation of Growth by Cell Division

The stimulation of rapid cell division and growth of plant cells is rarely simple, for no single compound unlocks the door of cell division. For instance, Braun (12) and Braun & Naf (13) found that two growth factors interact to determine the growth of tumors. Coconut milk, horse-chestnut endosperm, and corn extract comprise within each source a complex of substances which individually may have no activity, or at best only a portion of the activity of the whole. The requirement for reduced nitrogen compounds, in addition to the growth stimulatory substances per se, has been described by Shantz & Steward (93).

Even such active complexes as whole coconut milk may be without effect on some cells unless supplemented by additional growth-regulating compounds. This was first observed by Steward & Caplin (97) who found that potato tuber tissue could be cultured only by the addition of both coconut

milk and 2,4-D, both supplements being continuously needed (106). Following these suggestions, Letham (51) has induced growth in tissue of immature apple fruits by the use of similar mixtures of growth substances.

Shantz, Steward *et al.* used the potato tuber tissue system to assay substances that are synergistic with coconut milk and to determine the effect of the structure of synthetic growth-regulating compounds upon this interaction. Steward *et al.* (104) have also used the carrot assay system as a means to study substances that competitively inhibit the coconut milk effect. The effects which have been investigated are those of aromatic substitution in the phenoxyacetic acids (106, 108) and the phenylacetic acids (108), of optical isomerisms in the β-naphthoxypropionic acids (89), and of the length of the side chain in the phenoxyaliphatic acids (108) upon the interaction with coconut milk to produce growth. The inhibitory effects that have been investigated most are those due to a range of compounds related to proline or hydroxyproline (104). All these data emphasize the great specificity of these synergistic interactions with coconut milk in the induction or suppression of growth, and they emphasize how sensitive a regulatory control could be achieved by the balanced effect of synergists with the growth factors proper.

The notable conclusions are (*a*) substitution in the 2-position of the phenoxy acids seems especially conducive to cell enlargement, in the 4-position to cell division, while in the phenylacetic series substitution in the 4-position makes the compound inactive; (*b*) one substance may be an effective synergist to promote growth, and its optical enantiomorph may be competitively antagonistic to it; (*c*) the side chains with even numbers of carbons show activity, the odd ones are inactive following Wain & Wightman's concept of β-oxidation (128).

After the first recognition that proline is directly incorporated into a special protein moiety in the proliferating cells (96a) and is subsequently converted to hydroxyproline (103), it was found that hydroxy-L-proline, by acting as a competitive proline antimetabolite, is itself an inhibitor of the coconut milk effect (104). Other substances are less effective than hydroxy-L-proline in this respect (104). The D compounds are less effective than the L compounds; the allohydroxy-L-proline and substituted hydroxyprolines less than the unsubstituted ones; and the effects of N-substitutions being more pronounced than O-substitution. A substance like azetidine-2-carboxylic acid found in the Liliaceae (30), also acts as a proline antagonist (104); this shows how some unusual nitrogen compounds of plants could act as "blocks" to control growth in storage organs.

The kinetin of Miller *et al.* (61), 6-furfurylaminopurine, stimulates cell division in certain plant tissues, but is only effective in combination with IAA. Das *et al.* (18) found IAA to be indispensable in the stimulation by kinetin of mitosis in tobacco pith tissue, while Patau *et al.* (73) observed a similar requirement as measured by deoxyribonucleic acid synthesis in the same tissue.

Shantz, Mears & Steward (86) showed that some analogues of kinetin

are more effective than kinetin itself in stimulating the growth of carrot tissue cultures; the degree of activity, as measured by ability to induce cell division, is apparently very dependent on structure. Again, these compounds were active only in the presence of IAA, either substance alone being without significant effect. A number of the 6-substituted purines gave no growth unless casein hydrolysate, as well as IAA, was added to the medium. Schrank (82), on the other hand, tested the ability of various substituted purines to increase coleoptile elongation in the presence of IAA and reported that the main requirement for activity appeared to be a nonspecific substituent in the 6-position. A comprehensive review by Strong (116) on the chemistry and biochemistry of kinetin and related compounds has recently appeared.

The authors have found gibberellic acid to give very little promotion of growth in cultures of potato and carrot tissue. Similar results were obtained by Henderson (42) on *Helianthus* tissue cultures, while Nickell (68) observed that some tissue cultures responded slightly while others were unaffected. Bergmann (6), however, found gibberellic acid to be inhibitory to the growth of freshly explanted carrot tissue, but stimulatory to later subcultures. Therefore, gibberellic acid, like kinetin, may produce a major response in tissue cultures or systems other than an intact plant, if it can be used in some as yet unknown, subtle combination with other substances. In this respect, it is interesting to note that Skinner *et al.* (94, 95) found combinations of gibberellic acid and certain 6-substituted purines to promote seed germination.

It is clear, therefore, that the isolation and chemical identification of the natural growth-regulating substances is not only becoming more and more difficult by virtue of their number, but the biological detection and assay of their effects is also being complicated by the interactions which are being revealed. To conclude that a given substance, fraction, or extract has or has not significant activity may now require a far larger number of tests and combinations than was hitherto thought necessary.

Concluding Remarks

In the study of growth-promoting effects in extracts of endosperm and other organs, contemporary plant physiologists are still in a somewhat similar position to that of the animal physiologists of some thirty or forty years ago. Although a few of the accessory growth substances for animals and bacteria were then known, the present understanding of the numerous vitamins found in such potent sources as yeast extract required many years of painstaking biochemical work. This progress has been marked, however, by two features that plant physiologists should emulate, now that their somewhat parallel problem can be recognized. First, quantitative, specific, and rapid assays were developed wherever possible; second, the active substances were isolated, critically purified, and identified. Much aid came from the recognition that the results of work on vitamins and hormones in bacteria and animals were important to medicine and to veterinary science. The time

has now come to give a similar emphasis to work that can be done with higher plants. Sufficient evidence has been given in this review to show the widespread occurrence of the phenomena of growth induction by non-nutrient substances, to emphasize the great variety of naturally occurring substances that elicit these effects, and to show that synergistic systems of inhibitors and promoters in their balanced effect can furnish a system of chemical regulatory control of growth by cell division and by cell enlargement. In pursuing the investigation of these problems, it is now essential to maintain the closest contact between the biochemistry necessary for the identification of the substances and the morphology necessary to understand the milieu in which they occur and act.

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AUXINS AND FUNGI[1,2]

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Although the relationship of auxins[3] to fungi[4] has been investigated almost since the existence of phytohormones was first demonstrated in higher plants, it has received relatively little attention from reviewers. Boysen-Jensen (39a) and Went & Thimann (255) gave brief accounts of the information then available, and Janke (97) published the last general review in 1939. Since that time some aspects of the topic have been reviewed separately, especially those concerned also with higher plants. For instance, Melin (131) and Slankis (212) discussed auxin in relation to mycorrhizal associations, and Allen (4) summarized some of the work on auxin production by phytopathogenic fungi, a topic which was also discussed by Brian (42) with particular regard to the causation of pathological symptoms. A brief account of the use of synthetic auxins in the control of storage diseases was given by Marth & Mitchell (125). Ray (191) has discussed auxin destruction by fungi in his recent comprehensive review of the general topic, but it should be noted that destruction of IAA has so far only been demonstrated clearly in two species of Hymenomycetes.

The gibberellins differ from auxins in their effects on plant growth and will only be mentioned incidentally in this paper. The literature on these important fungal products has been thoroughly reviewed by Stowe & Yamaki (220), and Stodola (218) has prepared an extensive annotated bibliography.

AUXIN PRODUCTION BY FUNGI IN PURE CULTURE

General Remarks

Soon after the discovery in higher plants of a growth hormone, auxin, attempts were made to isolate the substance involved. Sources other than higher plants were tested, and Nielsen reported in 1928 (160) that the agar,

[1] The survey of literature pertaining to this review was concluded September 1958.

[2] The following abbreviations will be used: IAA (indole-3-acetic acid); IAN (indole-3-acetonitrile); IBA (indole-3-butyric acid); IPrA (indole-3-propionic acid); 2,3-D, 2,4-D, 2,5-D (dichlorophenoxyacetic acids); 2,4,5-T, 2,4,6-T (trichlorophenoxyacetic acids); MCPA (2-methyl-4-chlorophenoxyacetic acid); NAA (naphthaleneacetic acid); TIBA (2,3,5-triiodobenzoic acid).

[3] The term "auxin" or "phytohormone" designates substances active in higher plants as defined by Thimann (230) and Van Overbeek *et al.* (177).

[4] The names of fungi used in the original publications have been retained unchanged in the present review.

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after growth of *Rhizopus suinus* and *Absidia ramosa*, contained a substance which promoted the growth of *Avena* coleoptiles. During the next 30 years, auxin production was demonstrated for 30 of 34 genera and for 67 (89 per cent) of 75 species of fungi (including some varieties of *Fusarium*).[6]

Roberts & Roberts (193) tested several unidentified soil fungi with the *Avena* curvature test and found that only 46 per cent of 39 synthesized auxin on beef extract-peptone; none of 15 synthesized auxin on a medium without tryptophan. Brian (42) mentioned that none of 25 species produced more than a trace of auxin without added tryptophan, but that auxin was produced by all 25 species with tryptophan.

Table I lists the genera and species which have been examined for auxin production and indicates the material tested. It also presents the essential information regarding nutrient media, methods of detection and identification, and briefly mentions extraction methods if other than crude preparations were used. The few cases in which the extracts were fractionated are also noted.

Detection of Auxin

The *Avena* curvature test (called *Avena* test in the following) has been used almost universally and often exclusively to detect auxin produced by fungi; the straight growth test with *Avena* coleoptile or pea stem sections and the split pea stem curvature test have been favored only in recent years. Growth promotion of stems of intact plants, which could be due to gibberellins rather than auxins, has not been used as a test for auxin in fungi. Inhibition of root growth was regarded as one of the positive tests for auxin in culture filtrates of *Rhizopus suinus* (161), *Taphrina cerasi* (83), and *Clitocybe maxima* (126), and was the sole bioassay in one study of auxin in commercial penicillin (19). Root inhibition, however, can no longer be accepted as proof of auxin production since unrelated substances, including toxins, might have the same effect. For instance, a substance produced by *Aspergillus niger*, which differed from IAA on chromatograms, caused strong curvatures of corn roots and also considerable growth inhibition, to judge from the photograph (54). Auxins promote root formation, and Thimann & Went (227) observed rooting in culture media of *Rhizopus suinus*. This response has not been used specifically for auxin detection in fungi and can be induced by other substances, such as sugars, vitamins, and inorganic salts (229). There are reports on root initiation in cut shoots by culture filtrates of *Fusarium lycopersici*, *F. moniliforme*, *F. oxysporum* (136, 137), and *F. vasinfectum* (102, 136); but the active factor(s) has not yet been identified. The crude culture filtrate of *Penicillium* sp. induced rooting in *Ligustrum*, but IAA failed to give the response in contrast to NAA, its amide, and IBA (13). Recently Hirata (89) showed that a number of Fusaria synthesized both auxin and

[6] In this number the unidentified isolates of *Penicillium* are counted as one, and those of *Exobasidium* (88) are excluded.

TABLE I

AUXIN PRODUCTION BY FUNGI IN PURE CULTURE*

Organism†	Media‡ and main N source	Methods of detection and identification. Remarks on extraction, purification, and on individual components of media§	Auxin‖	References
PHYCOMYCETES				
Blastocladiales				
Allomyces arbuscula	S.s. (methionine)	A.c.t.; c.m., and E.e. of my.	+	123
Peronosporales				
Pythium mamillatum	S.s. (NO_3)	A.c.t.	+	195
P. intermedium	S.s. (NO_3)	A.c.t.	+	195
P. splendens	S.s. (NO_3)	A.c.t.	−	195
P. debaryanum	S.s. (NO_3)	A.c.t.	−	195
Mucorales				
Absidia ramosa	Malt a.	A.c.t.	+	160
	Malt s.	A.c.t.	−	160
Phycomyces nitens	?	E.e. of sp.; A.c.t.; M.W. ca. 175.	+(IAA)	85
P. blakesleeanus	Rice	E.e. of sp. in dark; fraction; A.c.t.; M.W. 165–8. M.W. active residue after boiling in HCl, aver. 330.	+(IAA) ("auxin a"?)	110
P. blakesleeanus	S.s. (asparagine)	E.e. of c.m., my, and sp. in dark, in cold; fraction.; p. chrom., A.c.t., activity only at R_f = IAA; color tests (see text).	+(IAA)	78
P. blakesleeanus	Pot. s.	Sp.; same as for S.s.	+(IAA)	78
Rhizopus suinus	Malt a.	A.c.t.	+	160, 161
	Malt s.	A.c.t.; with filter paper;	+	161
		Without filter paper.	−	160, 161
R. suinus	S.s. (NH_4-tartrate)	My. press juice; grown on filter paper; A.c.t.	+	161
R. suinus	S.s. (NH_4) on filter paper	Crude and E.e.; A.c.t.; Nielsen: also curv. of other seedlings, and root inhib. With NH_4-tartrate (also without filter paper).	+	29, 61, 161

* All tests were made on the culture medium after growth of the fungus (c.m.) unless other materials are indicated in the third column.

† Abbreviations: Numerals in ( ) after the name indicate the number of isolates of the same species tested. Diseases or disease symptoms caused by some of the pathogenic fungi are indicated in parentheses.

‡ Abreviations: S. = synthetic medium; s. = solution; a. = agar; pot. = potato medium; tryp = tryptophane; yeast, malt = yeast or malt extracts.

§ Abbreviations: c.m. = culture medium; my. = vegetative mycelium or mycelium with attached reproductive structures; sp. = sporangiophores or sporophores; A.c.t. = *Avena* curvature test; E. = ether; e. = extract(s); M.W. = molecular weight; M.P. = melting point; p.chrom. = paper chromatography; Salk., Ehr., nitrite = Salkowski, Ehrlich or nitrite reagents. Other abbreviations are self-explanatory.

‖ Abbreviations and remarks: + = auxin present; − = no auxin detected (where average *Avena* curvatures are given (e.g. 88, 89), values of less than 2° are not regarded as evidence for auxin); (IAA) = IAA was isolated or identified, or the evidence was sufficient to suggest the presence of IAA; a question mark signifies that the evidence presented is insufficient proof for or against the presence of auxin.

Organism†	Media‡ and main N source	Methods of detection and identification. Remarks on extraction, purification, and on individual components of media.§	Auxin‖	References
		With NH_4–PO_4, –SO_4, –Cl, or asparagine.	–	161
		With NH_4 salts of organic acids, –H_2PO_4, –HPO_4, and –Cl (buffered).	+	29
		NH_4Cl (unbuffered).	–	29
R. suinus	Peptone	A.c.t.	+	30
	s. & a.	E.e.; A.c.t.	+	188, 226
R. suinus	Peptone s.	E.e.; fraction.; crystn. of IAA; A.c.t.; color tests; M.P. ca. 160°C.	+(IAA)	228
R. delemar, R. reflexus, R. tritici	Malt ? s.	A.c.t.	+	107, 108
R. nigricans	Malt s. in sand	E.e.; fraction.; A.c.t.; M.W. 176 and 190.	+(IAA)	109
Sporodinia grandis	Malt s. & a.	A.c.t.	–	160
ASCOMYCETES				
Helotiales				
Tympanis sp.	Malt a.	Dried, powdered my, in lanolin on decapd. lupine hypocotyls.	+	101
Taphrinales				
Taphrina deformans	?	E.e.; red with $FeCl_3$–HCl.	+?	118
T. deformans (leafcurl),	Pot.s.	A.c.t.; *T.d.* also on a.	+	88
& *T. betulae* (blister)	S.s. (NO_3)	A.c.t.	+	88
	S.s. (tryp)	A.c.t.; *T.d.*, purple Salk.	+	88
T. pruni (plum pocket)	Pot.s.	A.c.t.	–	88
& *T. cerasi* (witches'	S.s. (NO_3)	A.c.t.	+	88
broom)	S.s. (tryp)	A.c.t.; *T.p.*, red Salk.	+	88
T. cerasi	Pot. a.	E.e.; A.c.t.; root inhib.	+	83
T. osumundae	Pot. s.	A.c.t.	+	88
(blister)	S.s. (NO_3)	A.c.t.	–	88
	S.s. (tryp)	A.c.t.; pink Salk.	+	88
Sphaeriales				
Graphium ulmi (Dutch elm disease)	?	A.c.t.	+	195
Ceratostomella ulmi (Dutch elm disease)	Peptone + tryp.	Pea stem sections and split pea-sections.	+	17
Ceratocystis fagacearum	S.s. (NO_3)	Split pea-sections.	+	68
(oak wilt)	S.s. (NO_3) + yeast e.	Split pea-sections.	+	68
Ophiobolus graminis	S.s. (NO_3)	A.c.t.	+	195
Glomerella cingulata	S.s. (NO_3)	A.c.t.	+	195
Hypocreales				
Nectria galligena (tumors)	S.s. (NO_3)	*n*-Butanol or E.e. of c.m. and my.; A.c.t.; tendril curv.	+	21, 24
N. cinnabarina	S.s. (NO_3)	As for *N. galligena*.	–	21, 24
Gibberella fujikuroi	Pot.s. and a.	A.c.t.	+	89
G. lateritium (10)	Pot.s. and a.	A.c.t.	+	89
G. lateritium (1)	S.s. (NO_3)	A.c.t.; lilac Salk.	+	89
	S.s. (tryp)	A.c.t.; brown Salk.	+	89
G. zeae (2)	Pot.s. and a.	A.c.t.	+	89

Organism†	Media‡ and main N source	Methods of detection and identification. Remarks on extraction, purification, and on individual components of media.§	Auxin‖	References
Aspergillales				
Aspergillus niger	Malt S. and a.	A.c.t.	−	38, 160
A. niger	Peptone s.	Crude and E.e. (38, 39), A.c.t.	+	38, 39, 109, 226
A. niger	Peptone s.	A.c.t., only without glucose, sucrose, etc.	+	198
		With mannitol and lactose.	+	198
A. niger	Other complex N, sources	A.c.t.; casein, fibrin, hemoglobin, yeast RNA.	+	38, 198
		Egg albumin	−	38
A. niger	S.s. (NO_3)	A.c.t.	−	38, 198
A. niger	S.s. (NH_4-tartrate)	A.c.t.	−	38
A. niger	S.s. (NH_4, NO_3)	E.e.; A.c.t.; with or without tyrosine.	+	222
A. niger	S.s. (amino acids)	A.c.t.; with histidine, leucine, lysine, phenylalanine, tryp., tyrosine.	+	39
		With alanine, arginine, asparagine, cystine, glycine, proline.	−	39
		With asparagine.	+	109
		With tyrosine; E.e.; fraction.; M.W. 169.	+(IAA)	109
A. niger	Corn steep liquor	E.e.; *Avena* sections; Salk. & Ehr. tests (see text).	−?	53
Penicillium sp.	Malt a.	A.c.t.	−	160
			+	104
Penicillium sp.	S.s. (NO_3)	Slit *Avena* sections.	+	14
Penicillin, crude or clinical	—	Root inhib.; red-violet with Hopkins-Cole reagent.	+?	19
		Purific.; crystn. of IAA; M.P. 167°C.	+(IAA)	47
Saccharomycetales				
Saccharomyces cerevisiae	s. (NH_4)	Cells; same as *Tympanis*, (Ascom.).	+	101
S. cerevisiae	—	E. & ethanol extr. of cells; A.c.t.	+	162
S. cerevisiae	—	Water e. & autolysis juice of cells; A.c.t.	+	107, 108, 109
S. cerevisiae	—	E.e. of cell plasmolysate; fraction; purific. of IAA; A.c.t.; red color with $FeCl_3$; M.P. 164–165; M.W. 193.	+(IAA)	109
S. cerevisiae	S.s. (NH_4)	C.m.; A.c.t.; with and without peptone.	+	194
BASIDIOMYCETES				
Ustilaginales				
Ustilago avenae	Malt a.	A.c.t.	+	104
U. antherarum, U. shiraiana, & U. onumae	Pot. s.	A.c.t.	+	88
	S.s. (NO_3)	A.c.t.	+	88
	S.s. (tryp)	A.c.t.; red or pink Salk.	+	88

Organism†	Media‡ and main N source	Methods of detection and identification. Remarks on extraction, purification, and on individual components of media.§	Auxin‖	References
U. hordei & *U. tritici*	Pot. s.	A.c.t.	+	88
	Pot. a.	A.c.t.	−	88
	S.s. (NO_3)	A.c.t.	+	88
	S.s. (tryp)	A.c.t.; red Salk.	+	88
U. nigra	S.s. (tryp)	Nitrite & Salk. tests.	−	261
U. nuda	Pot. s. & a.	A.c.t.	+	88
	S.s. (NO_3)	A.c.t.	+	88
	S.s. (tryp)	A.c.t.; red Salk.	+	88
U. zeae (4 strains) (galls)	Peptone tryp. s. & S.s. (inorganic N)	E.e. of cm. and my.; A.c.t.; c.m. and my. of all strains on both media (see text).	+	139
U. zeae	Malt a.	A.c.t.	+	104
U. zeae	Pot. s. & a.	A.c.t.	+	88
	S.s. (NO3)	A.c.t.	+	88
	S.s. (tryp)	A.c.t.; red Salk.	+	88
U. zeae	S.s. (NO_3)	No color with nitrite or Salk. reagents.	−	261
U. zeae	S.s. (tryp)	Crude or E.e.; red color with nitrite and Salk.; max. absorption at 530 mμ with nitrite; red spot with nitrite at R_f = IAA on p. chrom.	+(IAA)	261
U. zeae	S.s. + amino acids	No nitrite color with 19 amino acids, incl. tyrosine.	−	261
U. zeae	S.s. + tryptamine	Crude or E.e., no nitrite or Salk. color.	−	261
U. zeae	gelatin	No color with nitrite.	−	261
U. zeae	casein	No color with nitrite.	−	261
Uredinales				
Gymnosporangium juniperi-virginianae	S.s. (NO_3) + yeast e.	No color with Salk.	−	262
	S.s. (tryp) + yeast e.	Crude and E.e.; color with Salk. (absorption at 530 mμ); red nitrite spot at R_f = IAA.	+(IAA)	262
Puccinia graminis tritici	—	Cold ethanol e. of uredospores; p. chrom. of acid ether-soluble fraction; *Avena* sections, no activity at R_f = IAA, but at 2 other regions.	+(not IAA)	208
Puccinia graminis	—	E.e., circular p. chrom.; no IAA with Ehr.	−?	223
Agaricales				
Agaricus (*Psalliota*) *campestris*	—	Hot H_2O e. of fresh sp.; A.c.t.	+	163
A. campestris	—	Lamellae of fresh sp.; A.c.t.	+	242
Cultivated mushroom	—	Sp. (original paper unavailable).	+	37
Boletus edulis	—	Hot H_2O e. of dried sp.; ethanol & ether soluble; A.c.t.	+	163
B. edulis	—	Long-dried, and fresh sp.; A.c.t.	−	5
B. edulis	—	Freshly dried old and young sp.; A.c.t.	+	5
Clitocybe maxima	In soil	My.; A.c.t.; root inhib.	+	126
Polyporales				
Polyporus schweinitzii	Malt s.	My.; same as *Tympanis* (Ascom.).	+	101

Organism†	Media‡ and main N source	Methods of detection and identification. Remarks on extraction, purification, and on individual components of media.§	Auxin‖	References
Exobasidium camelliae (galls)	S.s. (NO_3)	Salk. negative.	−	260
	S.s. (tryp)	Salk. positive.	+?	260
E. camelliae, E. japonicum (galls), *E. pentasporium* (witches' broom), & *E. reticulatum* (blisters).	Pot. s. & a.	A.c.t. (except *E. reticulatum* on agar).	+	88
	S.s. (NO_3)	A.c.t.	+	88
	S.s. (tryp)	A.c.t.; red Salk.	+	88
E. gracile (2) (galls), *E. nudum* & *E. vexans* (blisters), *E. pieridis* (galls), & *E. symplocicaponicae* (galls).	Pot. s. & a.	Ac..t. (except *E. gracile* & *E. pieridis* on agar).	+	88
	S.s. (NO_3)	A.c.t.	−	88
	S.s. (tryp)	A.c.t.; pink or red Salk. (Salk. for *E. nudum*?).	+	88
Exobasidium sp., several isolates	Pot.s. & a.	A.c.t. Most.	+	88
	S.s. (NO_3)	A.c.t. Most.	−	88
	S.s. (tryp)	A.c.t.; pink or red Salk.	+	88
FUNGI IMPERFECTI				
Sphaeropsidales				
Ascochyta pisi	S.s. (NO_3)	A.c.t.	−	195
Diplodia natalensis	S.s. (asparagine)	Salk. text; with and without tryptophane.	+	73
Melanconiales				
Colletotrichum lindemuthianum	S.s. (NO_3)	A.c.t.	−	195
Moniliales				
Cercosporella herpotrichoides	S.s. (NO_3)	A.c.t.	+	195
Dematium pullulans	Malt s.	My.; same as *Tympanis* (Ascom.).	+	101
Helminthosporium sativum	S.s. (NO_3)	A.c.t.	−	195
Fusarium sp.	Malt s.	My.; same as *Tympanis* (Ascom.).	+	101
F. cucurbitae	Pot.s. & a.	A.c.t.; On agar, not in sol.	+	89
F. anguioides, F. cerosiae, F. oxysporum (3), *F.o.f. batatas, F.o.f. cucumerinum* (2), *F.o.f. lupini,* & *F.o.f. lycopersici.*	Pot.s. & a.	A.c.t.; In solution but none on agar.	+	89
F.o.f. nivium (4)	Pot.s. & a.	A.c.t.; (except 1 isolate each on agar and in sol.).	+	89
F.o.f. vasinfectum				
(1)	Pot.s. & a.	A.c.t.	−	89
	S.s. (NO_3)	A.c.t. (Salk. negative).	+	89
	S.s. (tryp)	A.c.t.; red Salk.	+	89
(2)	Pot.s. & a.	A.c.t.; (1 not on agar).	+	89
F. solani				
(1)	Pot.s. & a.	A.c.t.	+	89
	S.s. (NO_3)	A.c.t.; (but lilac Salk.).	−	89
	S.s. (tryp)	A.c.t.; brown Salk.	+	89
(3–4?)	Pot.s. & a.	A.c.t.; (except one isolate each on agar and in sol.).	+	89
Piricularia oryzae	S.s. (NO_3)	My.; slit pea sections; E.e.; p. chrom.; pink spot at R_f =IAA with Ehr.	+(IAA)	252

gibberellin. However, gibberellic acid did not promote rooting in cut shoots; instead, it inhibited root formation caused by IAA and NAA (42).

Considering that most workers have relied on a single test method and have worked almost invariably with crude or only partially purified material, the *Avena* test has the advantage of being the most selective and sensitive test for IAA and of being least affected by extraneous substances, such as sugars and inorganic ions which influence the growth of immersed coleoptile and stem sections (230, 255). The test is not specific for IAA because certain of its esters and other indole derivatives (including IAN), as well as certain unreleated compounds give curvatures, although mostly at higher concentrations than IAA (20, 255). Auxins other than IAA which are inactive or only weakly active in the *Avena* curvature test could easily be overlooked, and inhibitors produced by fungi could interfere with this and the other tests. Such interference with the IAA response by fungal products, including antibiotics, has been reported for the coleoptile section test (15). Another example is included in Curtis' recent work on *Aspergillus niger* filtrates. He found (53) that corn-steep liquor as well as potato-dextrose, Czapek-Dox, and Richard's medium, after growth of the fungus, induced the following abnormalities when applied to the apex of bean plants: stem thickening and twisting, tumors, inhibition of stem growth, and downward curvature of the stem and of the first compound leaves. Similar responses were obtained with other dicotyledons (55) but not with the aerial portions of corn or wheat seedlings (54, 55), although the *A. niger* factor caused curvatures and also stunting in corn roots (54). It was without effect when applied to bean leaves, and unilateral application to the stems did not produce negative curvatures in contrast to IAA (53). Malformations caused by the filtrate or by ether extracts could not be duplicated by a number of pure compounds including IAA, NAA, and IBA. Furthermore, ether extracts did not promote growth of *Avena* coleoptile sections. However, the unused medium (corn-steep liquor) did contain some auxin, and Curtis remarked (53) that preliminary evidence indicated inhibition of IAA-induced elongation by the *A. niger* factor. The factor chromatographed differently from IAA, and ether extracts of filtrates gave no color with the Salkowski and Ehrlich reagents after being spotted on filter paper. The substance was destroyed by boiling in 0.5 *N* NaOH, but not in 0.5 *N* HCl (53). Two strains of *A. niger*, but none of 37 other species of *Aspergillus* or 400 other unidentified fungi, gave the response. Curtis' evidence is sufficient to show that the substance, called "malformin" (55), differs from IAA, but it is not yet certain whether auxin is absent. Several workers (38, 39, 109, 198, 222, 226) reported earlier that *A. niger* grown on different nutrients produced auxin active in the *Avena* test, and the molecular weight was close to that of IAA (109). It would be worthwhile to study Curtis' two strains by means of the *Avena* test in conjunction with chromatography. Curtis was unable to detect "malformin" in a large number of fungi; but several years ago Mitchell & Angel (134) found that certain preparations of *Fusarium moniliforme* culture filtrates applied to the tip of the

primary leaf of bean plants occasionally gave a completely different response from the usual gibberellin overgrowth, namely stem twisting, inhibition of further growth, and formation of galls at the growing point. Although translocation through the leaf seemed to occur in this case the response resembles that given by the *A. niger* factor.

Other methods which have been used to detect auxin in fungi, with or without paper chromatography, include color tests for indole compounds by means of the nitrite, Ehrlich, and Salkowski reagents [the latter in several modifications, but primarily that of Gordon & Weber (75)]. The colorimetric or gross visual use of these reagents with crude culture solutions or extracts (see Table I) can hardly furnish reliable evidence for the presence or absence of auxin or give any information regarding its identity or quantity. Similar colors are obtained with several indole derivatives (219), and various colors, including purple, are given by the Salkowski reagent with phenolic compounds (219a). Interference by certain phenols at low concentration with the colorimetric Salkowski test for IAA is also known (180), and comparable phenomena could easily occur on chromatograms of insufficiently purified extracts. Other examples of interference with color tests for indoles and of their nonspecificity have also been reported.

To the above remarks must be added the comment that the presence of auxin may be overlooked if tests are made on filtrates from only one culture period. This is illustrated by Hirata's results (88) with species of *Exobasidium*, *Ustilago* and *Taphrina*, in some of which auxin was found in 15-day cultures but not in 30-day cultures.

The preceding remarks show that negative findings on auxin production by fungi must be viewed with considerable caution if based on single tests, and that the quantitative aspects of a majority of the positive findings are subject to grave doubt.

Identity of the Auxin

Nielsen determined some of the solubility characteristics of "rhizopin," the auxin produced by *Rhizopus suinus* (161). Its chemical and physical properties were studied further by Dolk & Thimann (61) who found the pK value to be 4.75, having recognized the compound as an acid. The molecular weight of the auxin from *Rhizopus nigricans* and *Aspergillus niger* (109) and from *Phycomyces* (85, 110) was found by the agar diffusion method to be close to that of IAA, i.e., 175. Isolation of crystalline IAA was reported almost simultaneously from yeast by Kögl & Kostermans (109) and from *Rhizopus suinus* by Thimann (228). No subsequent isolation of pure auxin from fungi has been reported, except that Cram & Tishler (47) obtained pure IAA as a contaminant of crude penicillin.

In recent years paper chromatography has been used to identify the auxin. Wolf obtained nitrite-positive spots at the R_f of IAA with ether extracts of culture filtrates of *Ustilago zeae* (261) and *Gymnosporangium*

juniperi-virginianae (262),[7] and Watanabe (252) obtained Ehrlich-positive spots corresponding to IAA from *Piricularia oryzae*. Gruen (78) detected auxin in the IAA region with the *Avena* test on chromatograms of separate ether extracts (acid fraction) of the culture filtrate, mycelium, and sporangiophores of *Phycomyces blakesleeanus* grown on synthetic medium and of sporangiophores grown on potato-dextrose medium. However, the Salkowski test showed IAA only on chromatograms with sporangiophore extracts from the potato medium and not with those from the synthetic medium which contained no tryptophan. Circumstantial evidence from estimates of the amounts present and of the sensitivity suggested interference by contaminants. The Ehrlich reagent gave weak spots in the IAA region with some of the material from the synthetic medium.

Although the auxin produced by other fungi (Table I) was detected mostly with the *Avena* test, the identification as IAA claimed by some workers cannot be regarded as well established in the absence of other tests.

Kögl & Verkaaik (110) reported that *Phycomyces blakesleeanus* sporangiophores grown on rice synthesized not only IAA but also auxin *a*, since the acid fraction from ether extracts retained 1/30 of its total activity even after boiling twice for 3 hr. in 5 per cent HCl. They observed no change in activity after the first period of boiling. The active residue gave an average molecular weight of 330 with the agar diffusion method (four determinations between 299 and 369). This is close to the theoretical value of 328 for auxin *a*. However, the existence of an auxin with the characteristics described by Kögl and co-workers for auxin *a* has not been confirmed by others and can no longer be seriously maintained (231). Recent studies by Wiedow-Pätzold & v. Guttenberg (256) showed that a single 3.5 hr. period of boiling in 1 *N* HCl only partially destroyed the auxin extracted from *Helianthus* hypocotyls, even when they had taken up IAA from solution and when the concentration in the extract was not supraoptimal for the *Avena* test. A second heating, however, completely destroyed the auxin indicating that only IAA was present. Kögl & Verkaaik's findings differ from those described by Wiedow-Pätzold *et al.*, and the consistently high molecular weight of the acid-resistant auxin in *Phycomyces* would suggest that it was not IAA, without proving, however, that "auxin *a*" was involved. Gruen (78) was unable to detect more than the one region of auxin activity at the R_f of IAA in sporangiophores of *P. blakesleeanus* despite the presence of Ehrlich- and Salkowski-positive spots elsewhere, but these results do not disprove Kögl & Verkaaik's findings since they used more extract.

Shaw & Hawkins (208) chromatographed ether extracts (acid fraction) of ungerminated uredospores of *Puccinia graminis tritici* and found no ac-

[7] Wolf obtained this saprophytic isolate of the normally obligate parasite from Cutter; it seems to represent a rare and probably abnormal form, at least as far as nutrition is concerned (56, 92). Neither Constabel (46) nor Turel & Ledingham (235) obtained any saprophytic growth of *Gymnosporangium juniperi-virginianae* or other Gymnosporangia (Constabel) from 1000 and more tissue cultures of galls.

tivity with the *Avena* section test in the IAA region, but, instead, in two other regions, one at a higher R_f (0.95), and one with less activity at a lower R_f (ca. 0.1). Van Sumere *et al.* (223) also failed to detect IAA with the Ehrlich reagent on chromatograms of an extract of uredospores from the same fungus.

The Effects of Environment and Nutritional Factors on Auxin Production

Nielsen (160, 161) was only able to detect auxin in cultures of *Absidia ramosa* and *Rhizopus suinus* on solid substrate, but Boysen-Jensen (38) found that *A. niger* produced auxin both on solid and liquid media. Bonner (29) and Dolk & Thimann (61) demonstrated that aeration was required for auxin production by *Rhizopus suinus* in liquid media.

The auxin content of the medium often changes markedly with time. Single peaks in auxin secretion were observed by several workers (29, 161, 188) and occurred at different times after inoculation, in part depending on the culture conditions including aeration (29) and temperature (188). A subsequent decrease in auxin concentration was also found (29, 188). On a constantly aerated liquid medium, Thimann & Dolk (226) obtained a slight peak in auxin secretion at approximately three days after inoculation (at the time of sporangium formation) and a maximum peak at about ten days, long after the mycelium had covered the surface of the solution. With more aeration an additional peak was found seven to eight days after inoculation. Bonner (29) had also observed that the peak in auxin production occurred more than two days after the maximum dry weight had been attained and had remained constant. According to Rabideau (188) the peak was accompanied by a decrease in dry weight and in acidity, which was thought to be due to autolysis. The *Avena* curvatures in this study were very small, 8° at the peak. Robinson & Stier's (194) curves for *Saccharomyces* culture media show two peaks, with the maximum not yet attained after 225 hr. The rate of auxin production was inversely related to the rate of cell multiplication, being highest when there was practically no growth. These changes with time in auxin level require further study.

The effect of pH on auxin production has not been adequately studied. The yield from *Rhizopus suinis* was not affected significantly by variations in the pH of the medium from 4.5–7 (29); but with *Saccharomyces* the highest yields were obtained at approximately pH 3 (194), and with *Aspergillus* acid conditions seem to be required (163).

Nielsen (161) reported that *R. suinus* secreted auxin when grown on malt extract or ammonium tartrate but not on other ammonium salts or on asparagine, even though the fungus grew on all these media. However, Bonner (29) did obtain auxin on three of the inorganic ammonium salts tested by Nielsen and also on ammonium salts of several organic acids, including two which gave very poor growth. According to Boysen-Jensen (39) *A. niger* produced auxin on synthetic media with several amino acids, including

tryptophan which was the poorest source and tyrosine which was the best. Kögl & Kostermans (109) also found tyrosine to be a good substrate for auxin production. Later workers who studied the effect of tryptophan reported that an external supply favored auxin production by *Rhizopus* and *Aspergillus* [tryptophan in peptone preparations (226, 228)], *Diplodia natalensis* (73), and by most of the species of *Taphrina*, *Ustilago* and *Exobasidium* tested (88). According to Wolf (261, 262), *Ustilago zeae* and *Gymnosporangium juniperi-virginianae* produced auxin only in the presence of tryptophan; the former produced none with tryptamine or with 19 amino acids, including tyrosine. However, only color tests were used in these studies, and both Moulton (139) and Hirata (88) found that *U. zeae* synthesized auxin (*Avena* test) on synthetic media without tryptophan. This is also true for a number of other fungi (Table I). The role of tryptophan as a precursor of IAA in fungi was first postulated by Thimann (228), but to date no steps in IAA biosynthesis from tryptophan have actually been established by experimentation. In effect, the work on the role of nitrogen nutrition in auxin synthesis can only be regarded as exploratory, and other nutrients have received even less attention. Magnesium influenced auxin production by *Rhizopus suinus* over a range of concentrations which did not affect the growth significantly (29). No auxin was detected by the *Avena* test with 0.04 gm./l. $MgSO_4$ or less. Calcium had no effect. Sakamura & Yanagihara (198) only detected auxin in the medium when *Aspergillus niger* was grown on peptone without sugars or glycerol, although growth was greatly stimulated by these compounds. Mannitol and lactose neither promoted growth nor inhibited auxin production. Unfortunately no actual data were presented. These findings, which are at variance with those of other workers, cannot be evaluated without additional study.

Almost all work on auxin production on different substrates is based upon tests of the culture medium (see Table I) and not upon auxin in the mycelium. It is possible that a small amount of auxin is synthesized in the body of the fungus while not enough is secreted into the medium for detection, particularly if crude solutions and short growth periods are used. One element common to several of the studies on the effects of nutritional factors, or on the time course of auxin production, is the absence of any correlation between auxin production (secretion) and growth.

AUXIN AND FUNGUS DISEASES OF HIGHER PLANTS

Auxin production by phytopathogenic fungi has attracted attention in relation to the pathological symptoms in higher plants. A number of pathogens (Table I) were tested for their ability to synthesize auxin in pure culture, but these findings taken alone are of limited value in their application to *in vivo* disease development. A different approach has consisted in comparing the auxin content of healthy and diseased plant parts, and in correlating the changes in auxin level with other factors altered by the pathogen.

Zimmermann (267), who was the first to explore this approach, briefly

reported that apices of *Euphorbia cyparissias* infected by *Uromyces pisi* contained ten times as much auxin as did healthy apices. Working with the same host and parasite, Pilet (183) claimed a one-hundredfold higher auxin concentration expressed as IAA in rusted (10^{-4} *M*) than in healthy (10^{-6} *M*) leaves. Similarly, leaves of *Sempervivum tectorum* infected with the rust *Endophyllum sempervivi* (182) contained up to a 1000 times more auxin than healthy leaves, 10^{-4} as against 10^{-7} *M*, in the leaf region where the parasite was localized and where the auxin concentration was highest. The quantitative aspects of these studies were recently criticized by Daly & Inman (57). IAA concentrations plotted against curvatures in Pilet's modified *Avena* test (181) show that $10^{-6}-10^{-3}$ *M* gave decreasing curvatures relative to the maximum of 20° for 10^{-6} *M* and that the curve below this maximum concentration is not a straight-line plot as in the classical test (255). Because Pilet did not mention whether diluted extracts of the diseased leaves were also tested, it is not clear to this reviewer how curvatures corresponding to 10^{-4} *M* were distinguished from those corresponding to 10^{-7} *M* or less. Besides this, the excess auxin levels reported by Pilet for diseased plants are considerably larger than those reported in other similar studies; and in Pilet's own recent publication (184) *Uromyces pisi*-parasitized *Euphorbia* leaves gave only 15 times more auxin than healthy leaves, comparing in his table the leaf regions giving the highest concentrations. The omission of tests on diluted extracts is also a defect in certain other studies which established quantitative differences by means of the *Avena* test.

Sukhorukov & Strogonov (222) found no auxin in *Avena sativa* infected by *Puccinia coronifera*, although slight amounts seemed to be present in healthy leaves. However, these results were based on *Avena* test curvatures of 2.2° measured after 4 hr. Hirata (86), using a modified *Avena* test, reported a thirteenfold increase in auxin in press juice of stems infected with *Uromyces alopecuri*. Only slight increases (1.6- and 5-fold) were measured in leaves infected with *Gymnosporangium haraeanum* and *Puccinia caricis-blepharicarpae*. Diffusates from the former gave only a very slight average curvature. Shaw & Hawkins (208) studied the auxin in two wheat varieties infected by stem rust (*Puccinia graminis tritici*) using the *Avena* section test on paper chromatograms of extracts. Diseased and healthy leaves of the susceptible variety gave auxin with the same R_f as IAA. After 11 days incubation the auxin content of the diseased leaves was about 20 times greater than in healthy leaves. After only five days incubation infected leaves of the other, resistant variety gave inhibition in the IAA region of the chromatograms and healthy leaves gave no definite activity. Parallel results were obtained with barley leaves infected by *Erysiphegraminis hordei*, with the same variety tested after five and ten days. Rust infection was also studied by Daly & Inman (57) with safflower hypocotyls infected by *Puccinia carthami*. The auxin in ether extracts of healthy and diseased hypocotyls was tested with the *Avena* section test after chromatography because less activity was obtained with unchromatographed extracts than after chromatography due

to inhibitor(s) which moved high on the strips. Diseased hypocotyls, tested from the eighth day of infection onward, showed more IAA on chromatograms than healthy ones; in one series of tests from twice as much on the eighth day to seven times as much on a fresh weight or ten times as much on a hypocotyl basis after 16 days. In healthy and diseased plants the auxin concentration reached a peak on the thirteenth day, possibly because of auxin movement into the hypocotyls from the first true leaves emerging at that time. The auxin in healthy plants corresponded to IAA; but in the early period of infection (sixth to eighth day) before the appearance of visible symptoms, the infected hypocotyls also contained other compounds giving growth activity, at least one above and a lesser one below the IAA region of the chromatograms. Most activity other than IAA was absent from healthy leaves, and during further development of the infection (tenth to thirteenth day) this activity disappeared from the chromatograms.

Smut infections also cause an increase in the auxin content. This was first demonstrated by Moulton (139) in *Ustilago zeae* tumors on corn leaf sheaths, which contained 16 times more auxin than did healthy leaves. Extracts of the mycelium of two tumor-forming strains of *U. zeae*, grown for long periods, produced more auxin (*Avena* test) both with and without tryptophan (with one exception) than did extracts of two strains which did not cause tumors. Wolf (261) reported that four strains of *U. zeae* in culture produced auxin with tryptophan in the medium (see above), but he could not detect auxin with the Salkowski and nitrite reagents in the tryptophan-containing medium of *U. nigra* which does not form galls. V. Guttenberg & Strutz (80) reported that the auxin from *U. zeae* galls was completely destroyed by the pea enzyme prepared according to Tang and Bonner. Press juice and diffusates from *Tuburcinia japonica* and *Ustilago onumae* galls yielded more auxin than did healthy tissues (modified *Avena* test) (86). Turian (237) chromatographed extracts of *U. zeae* tumors and did not detect any color in the IAA region with the Salkowski reagent. He concluded that IAA, if present at all, must have been less than 10^{-6} *M*, but he did not consider possible interference with the reagent.

Higher auxin levels in diseased plant parts than in healthy ones were also reported for infections by *Exobasidium*, *Protomyces inouyei*, and *Albugo candida* (86). Slices of a highly susceptible potato variety inoculated with *Phytophthora infestans* contained more auxin, and those of a resistant variety contained less auxin, than uninfected slices according to Sukhorukov & Strogonov (222). Again, however, all curvatures were measured after 4 hr. and were exceedingly low. The resistant variety was said to have a high "activity of the oxidases." Berducou (21, 24) detected auxin in the medium and mycelium of the tumor-inducing *Nectria galligena* in pure culture, but not in *N. cinnabarina* which does not cause tumors.

The findings discussed above raise questions regarding the source of the increased auxin level and its role in disease development. Baldacci (9) and Pilet (182, 183) have mentioned several hypotheses which could explain the

increase in auxin content of parasitized tissues. Recently, Pilet (184) showed by means of colorimetry with the Salkowski reagent that the rate of IAA inactivation in extracts of *Euphorbia cyparissias* leaves infected by *Uromyces pisi* is lower than in those of healthy leaves. The rate was lowest in regions bearing the largest number of aecidia, the inhibition reaching 61 per cent compared to the same region in healthy leaves. Although strongly infected leaves showed only feeble auxin inactivation, extracts of the aecidia alone destroyed IAA more rapidly than those of parasitized leaves. Pilet concluded that the fungus secreted a toxin which inhibited the IAA-destroying enzyme in the leaf tissue. Auxin inactivation in separate extracts of healthy leaves and aecidia decreased with increasing periods of incubation but less than in extracts of healthy leaves mixed with extracts of aecidia. Also, the inhibition of auxin destruction became more pronounced in the mixed solution as the concentration of the fungus extract was increased. Extracts of *U. pisi* aecidia inhibited auxin inactivation in extracts of other plants, and auxin destruction was also lowered in leaves of *Euphorbia verrucosa* infected by *U. scutellatus*. Turian (239) reported that the catalase activity in extracts of *E. cyparissias* leaves infected by *U. pisi* was greater than in healthy leaves, and he suggested that an increased inhibition of the peroxidasic phase of the IAA oxidase system might result which could favor an increase in auxin. Shaw & Hawkins (208) incubated leaf discs of wheat in carboxyl-labelled IAA and measured auxin destruction by the release of $C^{14}O_2$. Destruction of IAA was greater in leaves infected with *Puccinia graminis* than in uninfected leaves up to about three days after infection in the susceptible variety and up to four to seven days in the resistant variety. A few data for barley leaves infected by *Erysiphe graminis hordei* suggested a similar behavior. The corresponding changes in auxin level with time have not yet been explored sufficiently (see above), but the available data agree with the authors' hypothesis that the early decrease and subsequent increase in the auxin of diseased leaves are correlated with changes in the rate of auxin destruction.

However, it is difficult to decide whether auxin destruction observed in cut plant parts or in extracts of crushed material also takes place in the intact plant (191). Because the parasite injures the invaded tissues, an increase in auxin destruction could be expected and was actually observed by Shaw and Hawkins during early infection. Perhaps Pilet's findings apply to a later stage characterized by the accumulation of inhibitors which are either derived from the fungus or from the host itself. Shaw and Hawkins' work also suggests this. Phenolic compounds might be involved, and di- and polyphenols are known to inhibit auxin destruction by the *Omphalia flavida* enzyme (190).

Increased auxin synthesis by the host or auxin secretion by the parasite, could contribute to, or be solely responsible for, hyperauxiny in other diseases. Nothing is known about auxin production by the mycelium proper of the obligate parasites *Puccinia, Erysiphe* and *Uromyces;* but Pilet's data for

auxin destruction by the aecidia of *Uromyces* raise questions concerning auxin synthesis by these fungi. On the other hand, most of the facultative parasites produce auxin *in vitro* (Table I), particularly in the presence of tryptophan; and they will probably do so in the host, which would raise the auxin level. It may be noted that Hirata's work suggests that the changes in auxin level with time, in plants attacked by facultative parasites differ from the situation encountered in rust infections. The young galls due to *Exobasidium symploci-japonici*, *Tuburcinia japonica*, and *Ustilago onumae*, as well as *E. camelliae* and *Albugo candida*, contain initially more auxin than comparable healthy stem or leaf sections (86). But the diffusible auxin obtained from the galls of the first three species, and in some trials also from the last two, decreased markedly before the period of maximum growth of the galls (Went's *Avena* test was used) (87).

It should be recalled, too, that Shaw & Samborski (207) allowed leaves infected with *Puccinia* and *Erysiphe* (advanced infection) to take up carboxyl-labelled IAA, and they found that the radioactivity accumulated at the infection sites. The same observations were made with labelled protocatechuic acid and other substances. This shows that changes in translocation within the host might be induced by the parasite and cannot be ignored in studies on hyperauxiny.

Thus, while a beginning has been made in elucidating the causes of hyperauxiny, nothing definite is known regarding the role of auxin in fungus diseases. With regard to gall formation, the far more extensive work on crown gall (40) must be kept in mind. In the light of this information it is only possible to say that auxin probably participates in gall development but may not be the only factor involved, particularly in relation to the incitement of the galls. The available information is confusing. On the one hand, IAA (5 mg./l.) and extracts from *Rhizopus suinus* have been reported by La Rue (113a) to induce galls on *Populus* leaves; and, on the other hand, no galls are formed in some of the diseases discussed above in connection with hyperauxiny. Turel & Ledingham (235) reported that 0.1 mg./l. IAA in the presence of coconut milk stimulated callus and mycelial growth in cotyledon pieces infected by the rust *Melampsora lini*, while no effect was observed in the absence of coconut milk. Furthermore, it should be recalled that the tumor-inducing factor from *Aspergillus niger* differed from IAA (53, 54). Perhaps in some fungus diseases IAA acts only in the presence of other factors essential for tumor growth.

Other disease symptoms are equally difficult to interpret in terms of the increased auxin level. For instance, the leaf area and thickness underwent changes (183) and a doubling in leaf length resulted from infection with *Endophyllum sempervivi* (182). Daly & Inman (57) remarked that the leaf area of bean plants decreased with heavy infection (fungus?), and increased with moderate infection. Infected stems can increase in length (183), and *Puccinia carthami* caused some increase in the length of safflower hypocotyls (57). Because gibberellins induce elongation of intact stems and sometimes

also increases in leaf area (220), no decision can be made regarding the role of auxin. However, gibberellin may not be a frequent metabolite of fungi other than *Fusarium* (*Gibberella*), since Curtis (52) was unable to detect gibberellin activity in the culture filtrates of 98 genera and 251 species of fungi and in many other unidentified isolates (only one concentration was used). This does not preclude possible promotion of the production of gibberellin-like factors in the host. The observation (183) that *Uromyces pisi* causes premature leaf-drop in *Euphorbia* is unexpected; the opposite would have been anticipated in view of the higher auxin level in infected leaves. Premature leaf-drop has been reported as the result of infection with *Omphalia flavida*, which actively destroys auxin (205); and, since the leaves could be kept on by applying IAA, there seems little doubt that the auxin destruction is responsible.

Mycorrhiza constitute a special type of fungus infection in which auxin has been implicated in the formation of swellings and short dichotomously branched roots. In their early work with orchid mycorrhiza, MacDougal & Dufrenoy (122) believed that a red color obtained with the Salkowski reagent in cells invaded by the fungus and in the hyphae, but not in uninfected tissues, indicated the presence of auxin. But the color alone is no proof of the presence of auxin and could have been caused by other compounds. Slankis (209) obtained swellings and dichotomous branching in isolated pine roots after inoculation with the mycorrhizal fungi *Boletus variegatus* and *B. luteus* or by treatment with the culture media. This response could be strikingly imitated with higher concentrations of IAA (210) and NAA (211). Earlier, Björkman (28) had reported that IAA (2.5 and 25 μg./l.) had no effect on pine seedlings in sterile cultures infected with *Rhizopogon roseolus*, or without the fungus, and did not influence the formation of mycorrhiza in any way. However, the concentrations of IAA might have been too low, judging from Slankis' results. In connection with these studies, it must be pointed out that no tests for auxin have yet been made on the culture media of the mycorrhizal fungi (212).

The Effects of Auxins on Fungi

Effects on Growth

After auxin had been discovered in fungi it was thought that the phytohormone might play a role in their growth. However, Nielsen & Hartelius (164) failed to obtain any increase in dry weight yield of *Aspergillus niger* with auxin-containing ether extracts of *Rhizopus suinus* culture media and Boysen-Jensen (39) observed inhibition in *A. niger* when auxin concentrates from the same fungus were added to the medium. In the same year, Bonner (29) reported that increasing concentrations of auxin (not purified) from *R. suinus* did not affect the dry weight yield of this fungus. Subsequent work will be presented by grouping the test organisms in the different classes of fungi.

Phycomycetes.—"Auxin *a*" had no effect on *Pythium mamillatum* (195).

NAA either had no effect or inhibited growth at high concentrations in *Achlya sexualis* and *Saprolegnia ferax* (259). Similar overall results were reported for the following with IAA: *Achlya dubia, Pythiopsis intermedia, Aphanomyces camptostylus, A. cladogamus, Thraustotheca clavata* (140), *Achlya* sp., *Isoachlya anisospora* var. *indica, Saprolegnia monoica, Brevilegnia gracilis* (27), *Rhizopus suinus* (98, 204), *R. nigricans* (158, 241), *Pilobolus kleinii* (178), several other Mucoraceae (115), and *Phytophthora cactorum* (117a) which also failed to respond to IPrA and IBA. IAA, IBA, NAA, and 2,4-D had no positive effect with or without yeast extract on *Phytophthora cryptogea, P. parasitica, P. arecoe,* and *P. meadii* (129). High concentrations of 2,4-D inhibited *P. cactorum, Rhizopus nigricans* (51) and *R. oryzae* (124). The inhibition by 2,4-D, IBA, naphthoxyacetate, and 4-chlorophenoxyacetate was studied on *Mucor* sp. (234), by 2,4-D on *Mucor* sp. (62) and on *Pythium debaryanum* (26), and by 2,4-D, 2,4,5-T and MCPA on *Cunninghamella* sp. (159). The inhibition of *Cunninghamella* by the higher concentrations increased with increasing acidity, but inhibition by the ethyl esters of 2,4-D and MCPA was indifferent to pH (159).

Phycomyces is the only representative in this class which has been studied in detail. IAA (203) or "auxin" (249) alone was inactive as a growth factor, and there was no promotion with IAA in the presence of thiamine (98, 241) or yeast extract (115). Inhibition due to IAA was partially reversed by aqueous extracts of the fungus itself and of *Rhizopus* (241). According to Richards (192), growth of the mycelium of *P. blakesleeanus* on potato dextrose agar was promoted slightly (11 per cent) by 10^{-4} *M* IAA during early growth only, and 21 per cent by 1.8×10^{-4} *M* NAA during terminal growth. Other concentrations of these auxins as well as of 2,4-D and 2,4,5-T had no clear effect or were inhibitory; 1.8×10^{-3} *M* IAA inhibited growth completely. Inhibition by 2,4-D was also tested in *P. nitens* (51). Pohl & Rother (185) grew *P. blakesleeanus* on synthetic medium, and added IAA before or at intervals up to three days after inoculation. If added before inoculation, 5×10^{-4} *M* (88 mg./l.) IAA completely inhibited growth, 5×10^{-5} *M* greatly reduced growth, and 5×10^{-6} *M* or less had no effect. If added 12 hr. after inoculation, 5×10^{-4} *M* still caused total inhibition, but at 18 hr. only 6.3 per cent inhibition, and at three days almost none. The IAA inhibited spore germination (concluded at ca. 18 hr.) and early growth, but was ineffective when the mycelium reached the surface of the medium between two and three days after inoculation. The total inhibition by 5×10^{-4} *M* IAA added at the start was completely reversed by an aqueous extract from corn scutellum. The scutellum extract itself promoted growth if added within three days after inoculation (196); but with IAA, growth was the same as with the extract alone. Growth was never promoted above the controls in any experiment. "Indoleacetaldehyde" ($<10^{-5}$ *M*) and indoleethanol (5×10^{-5} *M*) had no effect on spore germination or growth, but they reversed completely the total inhibition by 5×10^{-5} *M* IAA. Benzaldehyde was inactive. The strain used for this work became more resistant to IAA after being maintained for

several months in darkness. Only early submerged growth, but no longer spore germination, was still inhibited by 5×10^{-4} *M* IAA; and total growth was only slightly less than the controls (15 per cent). Even 10^{-3} *M* gave only 23 per cent inhibition, which was reversible by the scutellum extract. Rother (196) chromatographed this extract and found two growth promoting regions for *Phycomyces*, one of which was identified as hypoxanthine, possibly together with guanine, and was the only region that reversed the IAA-induced inhibition. Pure hypoxanthine and guanine gave the same effect. However, the reversal of the weak inhibition in the resistant strain was regarded merely as compensation by growth promotion, and not actual antagonism as had been observed in the sensitive strain. An indole derivative supposedly present on the chromatograms of the extracts was apparently inactive. The authors suggested that the absence of inhibition of surface growth by IAA might be due to auxin destruction at the aerated surface. However, they ignored the earlier findings on IAA production by *Phycomyces*, and there is no evidence for IAA destruction.

A different approach was followed by Banbury (10, 11) who applied 10 mg. to 10 g./1. IAA in paste to one side of the growth zone of *P. blakesleeanus*, but did not obtain a curvature response. However, griseofulvin gave negative curvatures. In a preliminary study, Gruen (78) failed to obtain increases in curvature relative to water controls when drops of aqueous solutions containing 0.5 and 5 mg./l. IAA were applied unilaterally at different positions on the growth zone; 50 mg./l. gave inhibition, but only when applied fairly close to the sporangium and not further down on the growth zone. Indoleisobutyric acid (10^{-5}, 5×10^{-5} *M*) failed to give a response.

Ascomycetes.—No promotion of growth with auxins, or only inhibition, has been reported for the following species: with IAA, *Aspergillus niger* (43, 98, 195, 200), *A. candidus* (192), *Penicillium crustaceum* (241), *Penicillium* sp. (158) [also no effect of gibberellin; no measurements given (112)], *Hansenula anomala*, *H. saturnus*, *Mycoderma bordetii*, *Rhodotorula suganii*, *R. bronchialis*, *R. glutinis* var. *saitoi* (doubtful weak promotion, 19 per cent), *Torulopsis rotundata* (253), *Torula monosa* (253), *T. utilis* (158), *Cryptococcus neoformans*, *Candida albicans* (34), *Saccharomyces cerevisiae* (120, 200, 201, 253), *S. ellipsoides* (158), *Neurospora crassa* (*phen* mutant) (12), *Claviceps purpurea* (1, 74), *Ophiobolus graminis* ('auxin *a*') (195), *Ophiobolus herpotrichoides* (60), *Ophiostoma multiannulatum* (90), and three lichen fungi, *Placodiomyces saxicolae*, *Physciomyces pulverulentae*, and *Caloplacomyces aurentiacae* (265); with indole-3-carboxylic acid, *Cryptococcus neoformans* (202); with IBA, *Aspergillus niger* (200, 234); with IPrA, *Saccharomyces cerevisiae* (201), *Cryptococcus neoformans*, and *Candida albicans* (34); with NAA, *A. niger* (186, 200), *Ceratostomella ulmi* (1 gm./l.) (266), *Monilia albicans* (186), *Saccharomyces cerevisiae* (120); with 2,4-D, or its salts and esters, *A. niger* (62, 124, 144, 159, 186, 200, 234), *A. fumigatus* (18, 232). *Penicillium* sp. (51, 145, 214), *P. expansum* (51, 117), *P. notatum* (124, 214, 234), *Saccharomyces cerevisiae* (120, 200, 224), *Candida* (*Monilia*) *albicans* (34, 167,

186, 263), *Cryptococcus neoformans* (34, 202), *Gibberella saubinetii* (144), *G. zeae* (26), *Glomerella cingulata* (51, 144), *Ophiobolus miyabeanus* (232), *Sclerotinia sclerotiorum* (51, 144), *S. cinerea* (51), *Sclerotinia* sp. (246); with 2,4,5-T, *A. niger* (159), *Candida albicans*, *Cryptococcus neoformans* (34); and with MCPA, *A. niger* (159), and *Sclerotinia* sp. (246).

In contrast to these negative results, Kerl (103) reported strong promotion of mycelial growth (up to 176 per cent) and formation of apothecia in *Pyronema confluens* with a heteroauxin preparation in an inorganic medium in washed agar which supported very little growth alone. Apparently, no promotion was obtained on malt agar. The heteroauxin was very probably impure, but the maximum promotion given by "bios," "auxin," and thiamine was considerably less. Turfitt (240) found that IAA, α-NAA, and β-NAA (0.1–1 mg./l.) promoted growth of *Saccharomyces cerevisiae* in a synthetic medium. Promotion was noticeable after the first day; however, the auxin solutions were not fully sterilized before addition to the medium. The promotion given by both NAA isomers contrasts with the action of β-NAA as an auxin antagonist in roots (44). "Low concentrations" (not specified) of 2,4-D also promoted growth of *Saccharomyces* (213). Levan & Sandwall (116) obtained slight promotion of cell division in yeast with NAA but only during the first day. Scattered enlarged cells were found during the first day, but abundantly only at high, and even inhibitory concentrations. Bauch (16) first induced such giant yeast cells (gigas strains) with very high concentrations of IAA, IBA, and NAA, which, it was said, just failed to inhibit growth (1 g./l.!). Morquer & Nysterakis (135) obtained increases in colony diameter of *Candida albicans* with IAA (maximum with 50 mg./l.) over the controls on a synthetic medium, and inhibition with higher concentrations. The frequency of filamentization increased in the presence of auxin, most strongly at around 50 mg./l., but then decreased again with higher concentrations. Changes in cell dimensions were also observed. Scherr (201) noted increased cell multiplication and filamentization in *Saccharomyces* on NAA, but the number of viable cells decreased, indicating toxicity. One can hardly agree with the interpretation that filamentization or increase in cell size in yeast is an auxin effect, since it is obtained with unrelated toxic compounds (16, 116). Recently, Loveless *et al.* (120) reported increases in cell size in *Saccharomyces* at inhibitory concentrations of NAA, 2,4-D, and many other compounds. Haidasch (82) found that the dry weight yield of *Torula utilis* growing on wort was doubled by 1 mg./l. NAA. The fat content changed only slightly. NAA had no growth-promoting action on *Rhodotorula gracilis* on a synthetic medium, and was said to have none on *Torula* on that medium. 2,4-D (0.01 mg./l.) increased the yield of *Rhodotorula* by 40 per cent on wort, and a lesser promotion with an increase in fat content was obtained on synthetic medium. Other Ascomycetes which gave weak growth promotion (22 per cent at most) with auxins, at least during parts of their growth periods, include: *Aspergillus candidus* with NAA, and less with 2,4,5-T, *Neurospora tetrasperma* with IAA and 2,4-D (192), *Ophiobolus miyabeanus*

(144), *Sclerotinia oryzae-sativa*, and *Gibberella saubinetii* (232) with 2,4-D. Indole-3-aldehyde (100 mg./l.) added to a complex medium gave the highest dry weight yield of *Claviceps purpurea* compared to the basal medium, and to any other combination of compounds including IAA and other indoles (74). Indolepyruvic acid (and several compounds without auxin activity) supported normal growth of the *phen* mutant of *Neurospora crassa* (12).

Crowdy (48) found that several chlorophenoxy- and naphthoxy-acetic acids and IAA, IBA, and NAA at 100 mg./l. inhibited the growth of *Nectria galligena*, the indole auxins close to 80 per cent. Later, Berducou (22) reported that surface growth (colony area) of *N. cinnabarina*, which did not produce auxin (Table I), was slightly promoted (ca. 22 per cent) on a synthetic medium by close to 2 mg./l. IAA, slightly inhibited by 200 mg./l., and very strongly inhibited at about 1.2 g./l. In contrast, the auxin-producing *N. galligena* was slightly promoted (ca. 17 per cent) at 0.02 mg./l., greatly inhibited at 0.2 mg./l., and almost totally inhibited at 20 mg./l. A variant with abnormal morphology grew out on some cultures with 50 mg./l. IAA after two months (auxin content not tested) (23). Nothing further was reported on growth promotion in this fungus except for a remark (169) that up to 0.1 mg./l. promoted growth of *N. galligena*. However, Nysterakis discovered (168) that an autoclaved aqueous extract of carded cotton prevented even the total inhibition due to 400 mg./l. IAA on a synthetic medium. From 1 to 10 mg./l. of crude cotton extract, depending on the extraction method, reversed the inhibition due to 200 mg./l. IAA (170, 171). The extract alone promoted growth. Various experiments led to the conclusions that competitive inhibition was involved (169) and that the factor which reversed the IAA inhibition was probably identical with the factor giving growth promotion at low extract concentrations since any treatment changed the two effects in a parallel manner. The extract reversed the IAA-induced inhibition even if injected into an agar medium 15 days after inoculation, and apparently did not influence *Avena* curvatures given by IAA (171). After eliminating various possibilities, biotin was shown to reverse the IAA inhibition (170, 171), but in a quantitatively different manner than the cotton extract. Biotin had already been known to promote growth of *N. galligena* (25). A hypothesis proposed at that time to explain the role of biotin was recently upset by Nysterakis' finding (176) that citrulline also reversed the IAA-induced inhibition. Citrulline alone promoted growth at higher concentrations, but it reversed IAA inhibition almost completely even at a low concentration which did not promote growth. One factor which has not yet been taken into account in connection with the quantitative aspects of these studies is the IAA secretion by *N. galligena* itself.

Nysterakis extended his studies to *Neurospora tetrasperma* cultured on three different synthetic media, both solid and liquid (172). As much as 100 mg./l. IAA gave no inhibition, but instead 60 to 70 per cent promotion over the controls in two of the basal liquid media, and less in the third. Addition of agar (purified?) removed the promotion. Addition of cotton extract

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and biotin gave variable results, but mostly promotion relative to the basal medium with or without IAA. The two factors seemed to have a synergistic action in one medium. On agar the promotion by biotin and the cotton extract was considerably less than in liquid media. Thinking that the absence of inhibiton by IAA in *N. tetrasperma* might indicate excess production of the antiauxin factor, Nysterakis (174) tested the action of *Neurospora* extracts and intact autoclaved mycelia on *Nectria galligena* inhibited by IAA. *Neurospora* extracts removed the inhibition due to IAA, but extracts or mycelia of *Nectria* itself had only a slight effect. Quintin (187) reported that *Rhizopus nigricans* culture filtrates also reversed the inhibition of *N. galligena* by IAA in a manner similar to biotin which was detected in the filtrate.

Basidiomycetes.—Growth of *Ustilago zeae* was slightly promoted by IAA, apparently on a synthetic medium giving poor growth (96). Défago (60) found that *Tilletia tritici* (spore inoculation) was strongly inhibited in a nutrient solution with thiamine even by low concentrations of IAA. However, 6 mg./l. IAA, which gave a total inhibition in solution, promoted mycelial growth and increased the dry weight yield sixfold on agar containing basal medium without thiamine. The basal agar medium with thiamine alone gave better growth than with IAA alone. These results require further study, but the inhibition in solution might be comparable to the inhibition of spore germination and submerged growth by IAA which has been described for *Phycomyces* (see above). *Schizophyllum commune* was strongly inhibited by a wide range of concentrations of IAA, NAA, 2,4-D, and 2,4,5-T (192), but Naito & Tani (144) seemed to have studied a far more resistant strain with regard to 2,4-D. *Cortinellus shiitake* was also inhibited by 2,4-D (145), but slight growth promotion of 20 per cent or less was observed in *Corticium gramineum, C. centrifugum* (144), and *Hypochnus sasakii* (145). Only inhibition was observed in *Hypochnus centrifugus* and *Helicobasidium mompa* with 2,4-D (232). IAA had no positive effect on *Boletus variegatus, Clavaria dendroides* (130), and *Cortinellus berkelyanus* (158). Promotion of mycelial growth and dry weight by IAA and also by gibberellic acid (<10 mg./l.) were briefly mentioned (no measurements given) for *Cortinellus shiitake* (112). When added six days after inoculation IAA (10^{-3} to 10^{-6} *M*), IPrA, IBA, 2,4-D, 2-naphthoxyacetic acid, indole, skatole and tryptophan (all 10^{-4} *M*) were without distinct effect on *Polyporus versicolor*, but 10^{-5} *M* IAA added at the start was inhibitory. Added at the start, 10^{-4} to 10^{-5} *M* IAA did not influence *Marasmius scorodonius* while 10^{-3} *M* was strongly inhibitory (65). *Polyporus versicolor* is known to destroy IAA (65, 233), IPrA, and IBA but not indole, skatole, and tryptophan (233). When added after eight days growth 10^{-3} to 10^{-4} *M* IAA only slightly inhibited the growth of *Marasmius graminum* and *Stereum hirsutum* (66). Fries (71) reported that 0.4 mg./l. IAA, IPrA, IBA, and 4 mg./l. IPrA had no effect on the dry weight yield of *Lentinus omphalodes* in synthetic liquid medium; 4 mg./l. IAA strongly inhibited growth, and the same amount of IBA increased the yield with or without tryptophan which promoted growth considerably.

Indole also promoted growth. Four other Hymenomycetes, including *Lentinus lepideus*, were inhibited by tryptophan, and Fries thought that this might be due to conversion to IAA.

IAA (10μg./l.) slightly (significantly?) promoted growth of *Agaricus* (*Psalliota*) *campestris* f. *albida* on agar but only after 16 days incubation (35). IBA and 2,4-D had no effect, whereas IPrA was strongly inhibitory. Fraser (70) obtained different results with the same fungus depending on the culture method. Growth was poor on synthetic solution and was increased, though not sharply, by thiamine and biotin added together. IAA alone gave the same low increase in dry weight yield but, together with the two vitamins, promoted growth strongly over the controls with vitamins alone. This was true only if the inoculum floated on the surface; with immersed inoculum the promotion by IAA was abolished. The yields of surface growth were increased five to ten times by 5–100 mg./l. IAA and varied at random over the whole range of concentrations in a plateaulike response. However, 10 and 100 mg./l. of IAA, which promoted growth in solution, were strongly inhibitory on basal agar medium. Thiamine and biotin also no longer promoted growth on agar, but the combination of IAA with the vitamins was not tested. Yeast and cotton extracts and compost promoted growth in solution; the first two even submerged growth, but this promotion was not due to IAA. These results still require explanation, and one possibility suggested by Fraser, namely conversion of IAA to a growth factor, deserves further study. No growth promotion was observed with IAA in *Collybia velutipes* (7) though fruit body production was stimulated in some experiments.

The response to auxins has also been tested by direct application to the sporophores of agarics. Jeffreys & Greulach (99) applied agar blocks of IAA, NAA, and griseofulvin unilaterally to decapitated stipes of *Coprinus sterquilinus* and also sprayed unilaterally with IAA, NAA, griseofulvin, and with the auxin antagonist TIBA. The lower concentrations gave no response, while higher concentrations of IAA, NAA and TIBA gave positive curvatures (inhibition). Urayama (242) found that IAA and NAA, both at 0.01 and 1 g./l., had no effect when allowed to diffuse unilaterally into the cap of *Agaricus campestris*.

Fungi Imperfecti.—*Cercosporella herpotrichoides* showed essentially similar behavior towards IAA as *Tilletia tritici* (see above), namely inhibition in solution and promotion of growth (fourfold increase in dry weight) on minimal agar (60). A slight increase in colony diameter of *Alternaria tenuis* was observed with IAA (45); but several isolates of *Botrytis cinerea* tested with IAA, IBA, NAA and 2,4-D showed no consistent promotion (133). *Mycelium radicis atrovirens* was not promoted by 50 μg./l. IAA (130). Growth of *Fusarium oxysporum* f. *narcissi* was promoted with naphthalene acetamide and IBA (concentrations not given) (221). *F. oxysporum* var. *cubense* was inhibited by IAA and twice as strongly by IAN on a synthetic agar medium; but on a "starvation medium" of plain agar, a slight promotion (10 per cent or less) was observed (84). IAA (100 mg./l.) slightly promoted growth (23

per cent increase in dry weight) of *F. oxysporum* f. *lycopersici*, but the same concentration of 2,4-D, NAA, TIBA, and β-naphthoxyacetic acid gave no effect, or inhibition. Higher concentrations of these compounds were inhibitory, although IAA was without effect even at 500 mg./l. (58). The only effect of IAA, 2,4-D, and particularly IPrA, on *F. vasinfectum* was inhibition at high concentrations (31). Erratic results were reported for *F. oxysporum* f. *gladioli* with IBA and naphthaleneacetamide, but sometimes limited promotion resulted; also in one test on deficient medium (128). Reports on indifference to, or inhibition by, 2,4-D include the following species (other compounds tested at the same time are given in parentheses): *Alternaria solani* (117), *A. brassicae* var. *dauci* and *Alternaria* sp. (also MCPA) (246), *Botrytis* sp. (145, 246) *B. cinerea* (246), *Cephalosporium* sp. (also IAA, 2,4,5-T) (34), *Cephalothecium roseum* (144), *Colletotrichum phomoides* (51), *Epidermophyton inguinalis* (also IAA, 2,4,5-T) (34), *Fusarium dianthi* (124), *F. lycopersicum* (117), *Fusarium* sp. (214), [also 2,4,5-T, MCPA and their ethyl esters (159)], *Gloeosporium kaki* (144), *G. foliicolum* (145), *Helminthosporium turcicum* (51), *H. victoriae* (26), *H. sativum* and *H. teres* (246), *H. sigmoideum* (also some promotion) (145), *Macrosporium porri* (145), *Microsporum felineum* (also IAA; IPrA; 2,4,5-T), *M. audouini* (also IAA; IPrA; 2,3-D; 2,5-D; 2,4,5-T) (34), *M. canis* (*lanosum*) (18), *Ophiobolus graminis* (also MCPA) (111), *Pestalotia diospyri* (144), *Piricularia oryzae* (144, 232, 264), *Rhizoctonia tuliparum* (145), *Sclerotium delphinii*, *S. rolfsii* (51), *S. hydrophilum* (145), *Scopulariopsis brevicaulis*, *Sporotrichum schenkii* (also IAA; IPrA; 2,4,5-T) (34), *Trichoderma* sp. (also 2,4,5-T; MCPA and their ethyl esters) (159), *Trichophyton acuminatum*, *T. gypseum asteroides*, *T. granulosum*, *T. interdigitale* (also IAA; IPrA; 2,4,5-T) (34); *T. rubrum* (also IAA; IBA; IPrA; 2,3-D; 2,5,-D; 2,4,5-T) (34), *T. schoenleini*, *T. crateriforme*, *T. violaceum* (also IAA; IBA; IPrA; 2,5-D; 2,4,5-T) (32, 33, 34), *T. granulosum*, *T. gypseum*, *T. pedis*, *T. purpureum* (temporary immersion in 2,4-D solution, then growth on agar with some promotion found) (95), and *T. rosaceum* (first report on the action of 2,4-D on a fungus) (63). In addition to 2,4-D and NAA, various esters and other compounds were tested for inhibition on *Trichophyton gypseum* (186). The growth of *Sporotrichum schenckii* was not influenced by IAA (12.5 and 25 mg./l.) (166).

Helminthosporium sativum and *Rhizoctonia solani* were slightly promoted by MCPA and 2,4-D (amine salt) (246), and a maximum of 40 per cent promotion was observed in *Sclerotium oryzae-sativa* with 0.5 mg./l. 2,4-D (145).

Gentile & Klein (73) studied the growth of *Diplodia natalensis* on synthetic liquid medium with or without tryptophan and with the separate or combined addition of IAA and the auxin antagonist 2,4,6-T. Without tryptophan, 2.8×10^{-6} *M* IAA was produced after a week; but with tryptophan more auxin was obtained, for example $1.7\text{–}4\times10^{-5}$ *M* IAA with $1\text{–}5\times10^{-3}$ *M* tryptophan. Growth was inhibited by 14 and 25 per cent in these two tryptophan concentrations; and it was found that 10^{-10} to 10^{-9} *M* 2,4,6-T reversed the inhibition by 10^{-3} *M* of the amino acid or, rather, by the auxin

synthesized from it, to which the inhibition was attributed. The auxin antagonist itself inhibited growth at 10^{-5} to 10^{-4} *M*. Without tryptophan, 10^{-10} to 10^{-9} *M* 2,4,6-T had no effect on growth, and inhibition occurred with smaller amounts (10^{-6} *M*) than in the presence of tryptophan; 2,4,6-T did not interfere with IAA synthesis. Growth was then measured after adding both IAA and 2,4,6-T at inhibitory levels to the medium with or without tryptophan. Under these conditions, 2,4,6-T failed to reverse the inhibition due to IAA, but the latter reversed the inhibition due to 2,4,6-T within a certain range. The amount of 2,4,6-T which inhibited growth and that of IAA required to reverse the inhibition was higher with tryptophan than without, and this was thought to be due to the IAA synthesized from tryptophan. The contrast between the reversal of inhibition due to the tryptophan alone by low levels of 2,4,6-T and the absence of such reversal with added IAA still requires a definite explanation. No evidence was presented that the culture medium was purified or extracted before testing with the Gordon and Weber reagent, and the quantitative estimates of IAA are suspect unless possible interference by other fungal metabolites has been eliminated.

One of this reviewer's major objections is to the very basis of Gentile and Klein's study. Low concentrations of 2,4,6-T did not promote growth without tryptophan, and 2,4,6-T inhibited growth at a lower concentration without tryptophan present than with it. From this the authors concluded, as restated in their final discussion, that: "The results show that the endogenous IAA concentrations in the cultures are close to those required for optimal growth and that their biosynthesis is carefully regulated." Whatever the interaction of IAA (or tryptophan) and 2,4,6-T might mean, none of the results in the paper furnish any evidence whatsoever for a role of IAA in growth except as an inhibitor not necessarily different from other "poisons" either natural or synthetic for which antagonists may also be found. Nor has the "endogenous auxin" actually been studied, since IAA was not determined in the mycelium but in the culture medium where it accumulated. IAA was said to have inhibited growth after five days. The only evidence available is against a careful regulation of IAA biosynthesis, not for it. On the whole it appears unlikely that a positive growth-regulating role for IAA can be deduced from experiments with substances applied in toxic amounts.

Despite the numerous reports on inhibition of fungal growth by synthetic auxins, there have been few attempts to delimit the effective conditions. The influence of pH on inhibition by 2,4-D and related compounds was studied by Newman (159) and by Bouillenne & Bouillenne-Walrand (33, 34), who also included indole auxins in their work. Acid conditions favored inhibition by the acid compounds, probably because of the shift to the undissociated condition. Inhibition by the ethyl esters of 2,4,5-T and MCPA was indifferent to pH (159). In connection with these studies Bouillenne & Bouillenne-Walrand (33, 34, 36) found a contaminant in technical 2,4,5-T

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which also inhibited fungi and bacteria under alkaline conditions. A kinetic analysis of the inhibition of yeast by 2,4-D and IAA was recently presented by Frans *et al.* (69). No destruction of 2,4-D by fungi has been found so far (100, 248).

The only studies on the mechanism of action of 2,4-D have been carried out in Japan by Naito and his collaborators working with *Gloeosporium olivarum*. Apart from slight promotion of colony diameter (between 10 and 15 per cent) with 0.5–5 mg./l. on two of three media, 2,4-D gave primarily inhibition at higher concentrations. The inhibition was greatly increased by glucose and sucrose compared to a peptone medium without the sugars (143). Generally, more than 0.5 per cent glucose was required to give inhibition (dry weight) in the liquid peptone-inorganic salts medium, and the most pronounced effect was obtained with one to five per cent. It was shown that even at high concentration, 2,4-D only inhibited growth at pH 5.5 or less and that the inhibition increased with increasing acidity. Without 2,4-D the fungus was not affected by pH 4 to 8 (147). By growing the fungus on culture filtrates and ground mycelia obtained from previous cultures with or without 2,4-D, it could be shown (148, 155) that the inhibition was not due to 2,4-D itself but to an antibiotic produced as a result of exposure to 2,4-D. While the antibiotic is only produced at low pH, it is active also at pH 8. The possible effect of contaminants of 2,4-D was eliminated by the use of purified crystalline 2,4-D (154). The optimum concentration of the auxin for antibiotic production was 0.04 per cent. The substance is ether-extractable and was resistant to a wide pH range and to prolonged heating. Different media were tested as substrates (149, 154). The antibiotic was also revealed by the formation of an inhibition zone between colonies of *G. olivarum* on agar containing 2,4-D (151, 155). By fractionation and extraction, colorless needle-shaped crystals of the antibiotic were isolated (152, 154). Various characteristics (solubilities, color reactions, and chromatography) were tested, but no detailed chemical study has been published (152, 154). The effects of 2,4-D on the composition of the mycelium and on glucose and N uptake were studied, and it was found that uptake of the nutrients was increased despite the inhibition of growth (150).

An ether-extractable antibiotic was also found in *G. olivarum* cultures grown with 2,4,5-T and MCPA; but it was not found with NAA (K salt), a chlorophenol, or a phenylcarbamate (153). The antibiotic induced by MCPA was isolated as white needles with melting point 55°C., and contained C, H, O, Cl, and the OH group for alcohols but not N, P, or S. The solubilities and R_f of this compound agreed with the antibiotic induced by 2,4-D; but there are some differences, such as the melting point which was given for the 2,4-D-induced antibiotic only as an approximate figure (34°C.). Both antibiotics were inhibitory to several fungi and bacteria (156). These important results obtained by Naito and his collaborators should stimulate further work on the inhibition of other fungi by auxins, particularly synthetic auxins.

Auxins in Relation to Spore Germination

Windisch (257) was the first to study the effects of IAA on spore germination with *Bombardia lunata* ascospores. Slight promotion appeared in individual runs where the control values were low, but there was no overall effect except the usual inhibition at high concentrations. Acetic acid gave similar over-all results. Richards (192) found promotion of germination of *Aspergillus candidus* conidia with NAA (optimum: $3.16 \times 10^{-4} M$) but not with IAA, 2,4-D and 2,4,5-T. Nishikado & Inoue (165) reported considerable promotion of germination in *Gibberella zeae* conidia with IAA and 2,4-D after the fungus itself had been grown on media containing these auxins. IAA inhibited growth, but the length of the conidia and the number of septae were greater than on the cultures without IAA. Increase in size and number of conidia induced by MCPA has been reported for *Helminthosporium sativum* (246). According to v. Guttenberg and Strutz (80), IAA caused up to 75 per cent germination in *Ustilago zeae* spores while the controls in water did not germinate. Nicotinic acid also gave the same percentage of germination, but 2,4-D and 2,4,5-T were inhibitory. Van Sumere *et al.* (223) observed strong promotion of germination in *Puccinia graminis* uredospores treated with IAA, coumarin, protocatechuic acid, and other substances. The percentage promotion by IAA depended on the spore concentration since an increase in spore concentration led to a decrease in germination of the controls. With many spores IAA (200 mg./l.) increased the germination, but with few spores the same amount of IAA was actually inhibitory. It was suggested that IAA might reverse a self-inhibition of spores. Promotion of germination by 2,4-D was also reported (141) for *Gloeosporium olivarum* and increases in the number of sclerotia for other fungi (145). Wei & Ling (254) observed that low 2,4-D concentrations shortened slightly the time required for germination but did not increase the percentage of germination. The germination of meiosporangia of *Euallomyces* was promoted by IAA, IBA, indole and tryptophan according to Machlis & Ossia (123); and Turian (236) later reported promotion of germination of *Allomyces* zygotes by IAA.

On the other hand, only inhibition of germination at high concentrations of IAA, 2,4-D and several related compounds was observed in *Botrytis allii* (41), and in many cases germ tubes were stunted. IAA at several concentrations had no effect on *Saccharomyces cerevisiae* sporulation (132). The germination of uredospores of *Puccinia coronata* was not promoted by IAA or 2,4-D (179); and that of uredospores of four other rusts, including *P. graminis* was inhibited by 2,4-D and NAA (142). *Penicillium digitatum* conidia were inhibited with high concentrations of 2,4-D (79).

Miscellaneous Effects

Agar blocks containing IAA (0.04–2.5 mg./l.) or its methyl ester induced positive chemotaxis in the creeping plasmodia of *Didymium nigripes*

var. *xanthopus* (105). When placed near the front of the plasmodium, auxin-containing blocks increased the ratio of forward:backward movement of cytoplasm relative to controls without auxin (106); and IAA also increased the electrical potential difference between the front and back of the plasmodium (250) and between the front and the substrate (251).

Abnormalities and morphological changes caused by auxins, usually at high concentrations, include changes in colony morphology of *Phycomyces*, *Aspergillus*, *Neurospora* (192), *Schizophyllum* (157, 192), and *Gloeosporium* (157), variants in *Neurospora* (173, 175), increased sectoring in *Helminthosporium* (93) and *Pestalotia* (146), abnormal coremia in *Ceratostomella* on elm bark (67), decreased pigmentation in *Phycomyces* and *Neurospora* (192), and reduced formation of perithecia in *Neurospora* (192). The abnormally thin sporangiophores produced by *Phycomyces* on 10^{-3} *M* IAA remained phototropic (196).

Fermentation by *Saccharomyces* was said to be strongly stimulated by IAA, IBA, IPrA, and NAA (77); but Turfitt (240) found only slight stimulation with IAA and NAA. Inhibition of pyruvate oxidation was studied with high auxin concentrations (8). Anker (6) reported that IAA stimulated respiration in yeast, but this stimulation could have been due to adding auxin in alcoholic solution (6a). As well as inhibit fermentation (213, 224), 2,4-D can stimulate respiration and fermentation (224). Laccase formation was increased by IAA, IPrA, IBA in *Polyporus versicolor* (65); laccase formation was also increased slightly by IAA in two *Marasmius* species but not in *Stereum hirsutum* (66) or with IAA, NAA, 2,4-D in *Collybia velutipes* (121). Neither IAA nor related compounds induced alkaloid production in *Claviceps purpurea* according to de Tempe (225) and Gjerstadt & Ramstad (74), but Abe (2) reported some promotion. Citric acid production by *Aspergillus niger* was increased to some extent by IAA (247); and both IAA and NAA promoted penicillin formation (and growth?) by *P. notatum* on a simple medium, but less than on corn steep liquor alone in which the auxins were ineffective (114). Turian (238) found that the latex of *Euphorbia* parasitized by *Uromyces* had a higher acid phosphatase activity than the latex of healthy plants. Addition of 10^{-3} *M* IAA to healthy latex increased the phosphatase activity, and Turian thought that the increased auxin concentration in the diseased plants could have the same effect.

Disease Control with Auxins

The use of auxins in the treatment of fungus diseases can only be considered in a summary fashion. Thus, there was no effect of IAA on *Puccinia* infection (76), of various auxins in soil on a cotton seedling disease (189),of IBA and NAA on *Fusarium* scale rot of lilies (127), and of 2,4-D on *Melampsora* and *Septoria* on flax (197). Increased disease incidence after auxin treatment was observed with *Helminthosporium* on wheat (93), *Fusarium* on potatoes (64), *Narcissus* basal rot (221), *Claviceps* (119), and after 2,4,6-T treatment of *Didymella* on tomatoes (50). On the other hand, auxins and related compounds increased the resistance to bean chocolate spot (*Botrytis*

fabae) (49, 138), *Helminthosporium* on rice (3), *Fusarium* (245) and *Phytophthora* (206) on tomato, *Puccinia* (94, 258), citrus storage diseases (72, 79, 81, 113, 215 to 217), and *Verticillium* wilt of potatoes (243, 244). Healing of *Nectria galligena* cankers was obtained with IBA and other compounds (48). Köhler (111) carried out detailed studies on the effects of 2,4-D, its butyl ester, and IAA on plants infected with *Ophiobolus*, *Erysiphe*, *Ustilago*, *Tilletia*, and *Helminthosporium*. Working with *Fusarium* on tomato, Davis & Dimond (58) found that IAA, NAA, 2,4-D, naphthoxyacetic acid, and TIBA increased resistance at concentrations in the plant which must have been below the toxic level for the fungus in pure culture. Thus the disease resistance was attributed to changes in the host, and later studies (59) suggested that factors involved in resistance are present in the tops of tomato plants. The significance of the sugar content of the host in the response of diseased plants to 2,4-D was discussed by Horsfall & Dimond (91).

Although it has been shown that auxin production is widespread among fungi belonging to different taxonomic groups, more than 30 years' work has furnished no evidence for a growth-regulating role of auxins, particularly of IAA, in fungi. Assuming that these organisms have regulatory substances, synthesis must occur on any substrate permitting growth. Furthermore, a regulatory mechanism presupposes an internal balance of the active factors; and external application of such substances would probably give inhibition for the most part, or no response, unless the endogenous production center(s) are removed. While auxins do have such effects, their production seems to be poorly regulated. Their production is not related to growth and is quite variable in the same species, depending on the culture conditions. Auxin is often produced "in excess" since it accumulates in the substrate. These observations do not favor the view that auxins have a growth-regulating role in fungi. On the other hand, weak growth promotions of the order of 20 per cent by externally applied auxin have been noted frequently. These results are subject to doubt, since their statistical significance was not evaluated and because the methods used to measure growth, for example, the diameter or area of colonies, are not completely reliable. However, if we consider the cases of marked growth promotion with IAA, a common factor becomes apparent, namely that the promotion occurred on media which supported poor growth. This was true with *Pyronema* (103), *Tilletia* and *Cercosporella* (60), *Psalliota* (70), and with *Neurospora*. Regarding the latter, Nysterakis (172) already commented on the fact that IAA caused growth promotions, particularly on the basal liquid media which supported poor growth. Rather than emphasizing the inhibition by auxins, more attention should be given to these examples of growth promotion on "starvation media."

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PHOTOTROPISM AND PHOTOTAXIS[1,2]

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Introduction

It has been five years since Brauner (7) discussed the literature on tropistic and nastic movements in the *Annual Review of Plant Physiology*. A part of the newer literature on phototropism and phototaxis has been summarized elsewhere (14, 27, 36). In another recent review (67) attention was chiefly given to the publications up to 1950. The present report covers the period between 1954 and the beginning of 1958 and deals with light-induced movements in higher plants and fungi as well as phototactic movements of algae. Because the principal emphasis is placed upon features common to both topics, the rôle and nature of the pigments involved in the absorption of the light stimulus are first discussed. A further important point which is considered concerns the reactions by which this stimulus results in movement. In contrast with phototropism in higher plants, for which (through the intimate connection with research on auxins) a relatively broad empirical basis exists, only the first steps have as yet been taken with fungi and algae toward a clear understanding of the chain of reactions between light stimulus and movement.

Phototropism

Higher Plants.—During recent years the arguments in favor of the participation of carotenoids in phototropic movements have lost most of their basis. This applies especially to the concept of a carotene-mediated inactivation of auxin, which is mainly based upon the results of Kögl & Schuringa (42); in addition, the carotene-mediated lateral translocation of auxin, as postulated by Went (67), has also been questioned (26).

The assumption that riboflavin or flavoproteins are the actual sensitizers in phototropic movements and other growth reactions caused by blue light appears much better established. This new concept is mainly based on the accumulating data which indicate that IAA is the main auxin of higher plants and on the demonstration by Galston (25) of the strong sensitizing effect exerted by Rfl upon the photolysis of IAA. This concept has been further supported by experiments of the present writer (59, 60) showing that the carotenoids do not catalyze the *in vitro* destruction of IAA in light but

[1] The survey of literature pertaining to this review was concluded in June, 1958.

[2] The following abbreviations will be used: IAA (indole-3-acetic acid); 2,4-D (2,4-dichlorophenoxyacetic acid); Rfl (riboflavin; mc (metercandle); mcs (metercandle seconds).

[3] The author is greatly indebted to Dr. A. W. Galston and Dr. W. Haupt for valuable suggestions and to J. Landgraf for assistance in translation of the manuscript.

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protect this compound—probably by competitive absorption—against the action of sensitized Rfl. Through the interactions of the two pigments, the single peaked action spectrum of IAA photolysis by Rfl is transformed into a double-peaked curve. Although with intact organs, absorption and scattering of blue light by the cell walls (4000 to 4500 A) are certainly also involved, this protective effect of the carotenoids has been the basis of a hypothetical explanation (now commonly referred to as the filter theory) of several facts contradictory to the presumed function of Rfl in phototropism, namely: the resemblance of the double-peaked phototropic action spectrum of *Avena* coleoptiles to the absorption curve of carotenoids, the coincidence of the high phototropic sensitivity of the coleoptile tip with the accumulation of carotenoids in this region, and the difference between the strong light-growth reaction and the poor phototropic response of decapitated coleoptiles. Accordingly, in recent work, the role of Rfl in light-influenced movements and growth, the validity of the filter theory, and the status of the Cholodny-Went theory have been examined.

It can be stated as a generalization concerning recent investigations on the nature of photoreceptive pigments, that they conform almost exlusively with the assumption of a decisive function of the flavins and not of carotenoids in phototropism. Strong evidence against an active role of carotene in phototropic responses are observations by Labouriau & Galston (43). They found that virtually carotene-free coleoptiles of genetically albino barley and carotene-deficient *Phycomyces* sporangiophores in which carotene synthesis has been prevented by nutritional means, reacted to unilateral illumination by producing almost normal phototropic curvatures. Thereby, earlier results obtained with carotene-deficient maize coleoptiles (5) and *Phycomyces* (59) which led to the conclusion that carotenoids are not absolutely necessary for phototropic movements are confirmed.

With regard to a function of carotene as an internal filter, the as yet unestablished action spectra for the phototropic and light-growth reactions of carotene-free and normal coleoptiles will probably show interesting qualitative differences; in addition there may be quantitative changes in their phototropic sensitivity to different light intensities.

Investigations on the long neglected phototropic efficiency of ultraviolet light have led to results which as yet cannot be finally evaluated; nevertheless, there are indications that also in this region of the spectrum the flavins participate in the absorption of phototropically active radiation.

Mills & Schrank (50) studied the phototropic response of *Avena* seedlings to high intensity ultraviolet light (10^5 ergs/cm.^{2}). By using broad spectrum filters and irradiating the entire coleoptile flank, they succeeded in inducing positive curvatures which were strikingly similar to the base reaction in visible light. The short-wave range (2500 to 3500 A) proved most effective, whereas wavelengths between 3500 to 4000 A caused only feeble movements. If one considers the fact that from the broad spectrum of light utilized, two maxima of Rfl-absorption (2600 A and 3650 A) may have been encountered

at the shorter wavelengths and only one in the range above 3500 A, then these results correspond well with the presumed sensitizer function of this pigment. It should be further noted that the bending of the coleoptiles was clearly dependent on auxin supply, for after decapitation they reacted only when the tips were replaced by agar blocks containing IAA.

Curry, Thimann & Ray (23) report similar but not identical base curvatures in *Avena* seedlings after exposure to ultraviolet light of lower intensity (ca. $1\text{–}6\times10^2$ ergs/cm.^{2}). However they exclude any participation of Rfl at this energy level and conjecture that direct inactivation of an unknown bound form of IAA which controls growth is the cause of these movements. The main supports for this thesis are the close adherence of the action spectrum of the bending to the absorption spectrum of IAA and the strict dependence of the reaction of decapitated coleoptiles on IAA supply and rate of transport. Participation of free IAA is thought to be excluded because the action spectrum—compared to the IAA absorption spectrum—is minimally but unambiguously shifted (20 to 30 A) to longer wavelengths, and *in vitro* destruction of pure IAA requires much higher ultraviolet intensities than those necessary for the base curvature. Regarding the interpretation of Mills' & Schrank's results, two short remarks of Curry *et al.* on the effect of other energy levels deserve attention. Very high energy irradiation in the range around 3650 A (here almost no reaction occurs at the low energy level) produces bendings similar to the second positive curvature in visible light. A distinct tip reaction which has been observed at the same wavelength needs only a very low light intensity (10 ergs/cm.^{2}) and may be the counterpart of the first positive curvature.

Since several flavins have an absorption maximum at 3650 A and the carotenoid of *Avena* coleoptiles is, according to Karrer *et al.* (41), xanthophyll which shows only minimal absorption at this wavelength, it is almost certain that flavins are involved in these reactions. Nevertheless, just as for phototropism in visible light, the question remains open whether the difference in energy level required for curvature is associated with different photochemical mechanisms.

The starting point for an investigation by Mer (47) was the observations of Galston & Baker (28) on the differential behavior of etiolated pea stem sections in media containing either IAA or IAA plus Rfl. It was reported that the elongation of the segments was promoted by the pigment in darkness but inhibited in light. These effects have been explained by *in vitro* photolysis of IAA mediated by Rfl and *in vivo* interactions of absorbed Rfl with the native growth hormone. In an attempt to confirm these results Mer, using intact coleoptiles cut at the base and segments from wheat coleoptiles, showed that the light sensitivity of both objects could not be increased if their flavin level was raised by feeding with Rfl. In his experiments illumination with white light (100 to 500 ft.c.) also inhibited the growth of both coleoptiles and segments, and in a following dark period he observed an enhanced elongation of the highly uniformly reacting segments; but these

effects were the same in the controls (no addition of Rfl) as in the material with a raised flavin level. Hence Mer concludes that the pigment either did not penetrate the cells or failed to react with the native growth hormone. In further experiments with wheat segments immersed in solutions containing Rfl or IAA alone or Rfl plus IAA he observed that in light the strongest growth inhibition occurred with Rfl plus IAA. In the dark period following this illumination a stronger growth was found only in the medium containing IAA alone. Yet there were no significant differences for the increments of segments in Rfl or Rfl plus IAA and the controls which had been incubated without these compounds. Consequently, there is only one effect—*in vitro* photolysis of IAA leading to growth inhibition—which he ascribes to the action of the applied Rfl. Since finally a statistical evaluation of the experimental data for pea segments points to the same situation (no *in vivo* interaction of Rfl with native auxin) Mer reaches the conclusion that the most essential proof in favor of the function of flavins as photoreceptors, that is, *in vivo* destruction of native auxin, has yet to be demonstrated. He furthermore considers it doubtful that free Rfl is identical with native photoreceptors.

Interesting as the experimental findings are, there is no reason to overestimate the bearing of Mer's conclusions on phototropism. His most important statement, that there is no direct proof for *in vivo* interactions of light sensitized flavins with native auxin, may probably be right but it is not new. Several years before, Gordon (31) expressed the same opinion when he stated after a critical evaluation of the literature on this question: "It is doubtful whether endogenous photolysis of auxin is involved in the decreased growth response of etiolated tissues exposed to low light intensities." The second statement of Mer which excludes the participation of free Rfl in endogenous auxin inactivation is at present not sufficiently founded. The fact that the light sensitivity of coleoptiles and segments was unaltered after raising the Rfl level can be explained not only by the alternatives which Mer presents, i.e., either no penetration of the exogenously applied Rfl into the cells or no reaction between this pigment and the native hormone but also by the possibility that the objects used already had an optimal Rfl level. Further uptake of a relatively small quantity of this compound could then hardly result in an enhanced light response. In this connection, in etiolated bean hooks whose growth was not influenced by blue light, feeding with riboflavin phosphate, a flavin mononucleotide, led to an increase in the sensitivity to short-wave light (41a). Galston (27) recently reported that the growth of etiolated pea stem reactions was promoted in the dark by feeding with the same compound. Since, in these experiments, no exogenous IAA was applied the results obviously have *in vivo* significance.

A new way by which free Rfl may become active in the cell has been suggested by Waygood & MacLachlan (64). They found that the *in vitro* activity of IAA oxidase in darkness could be inhibited by Rfl or Rfl phosphate, and that this inhibition could be reversed with blue light. On the

basis of these and other results which suggest that similar conditions might apply for the action of this enzyme in cells they formulated a working hypothesis according to which Rfl or other flavins may *in vitro* continuously exert a check on IAA destruction, the extent of which is determined by illumination.

Further support in favor of flavins as the actual light receptors in phototropic reactions was obtained from experiments designed to clarify questions which arose in connection with the postulated filter function of carotenoids.

Thorning (63) investigated the relative efficiency of several wavelengths of light (4470, 4600, and 4770 A) on the phototropic bending of intact coleoptiles, as well as on the light-growth reaction of decapitated ones. The wavelengths selected are particularly interesting because they include the range of the two maxima (4500 and 4800 A) and the minimum (4600 A) of the phototropic action spectrum. Results with intact coleoptiles confirmed the familiar curves (28, 40) which are similar to the absorption curves of carotenoids. The wavelength of 4600 A was clearly least efficient. However, upon removal of the coleoptile tip containing the carotene a significant change was observed. With bilateral illumination the intensity of the resulting light-growth reaction was highest in short-wave light (4470 A) and diminished with increasing wavelengths. This corresponds exactly to the absorption of Rfl with a maximum at 4450 A, a fact which led Thorning to base her interpretation on the filter theory and to assume that the actual cause of the movements is the perception of the light stimulus by Rfl. Accordingly, she attributes the carotene-like phototropic action curve to a competitive absorption of blue light by the two pigments.

Bünning (13), who re-examined the distribution of the carotenoids in *Avena* coleoptiles, favors the view that the ratio of ~64 μg. xanthophyll/gm. dry weight to ~30 μg. Rfl/gm. dry weight found in the uppermost 2 mm. of the coleoptile tip (28) is sufficient to explain the double-peaked phototropic action spectrum, even though the movements are only produced by photochemical reactions sensitized by Rfl. He assigns to an unknown pigment in the coleoptile base the function served by the carotenoids in the tip. This pigment also strongly absorbs blue light. Slight differences in the action spectrum of the base reaction and of Rfl absorption are considered to be the result of interactions of the unknown pigment and Rfl. However, it could also be assumed that a direct relation exists between the base reaction and the unknown blue absorbing pigment, for its absorption curve and the action spectrum of the base reaction resemble each other strikingly.

In this connection, a paper from Goldacre (30) is also of interest. In dialysates of homogenized pea epicotyls, in addition to Rfl, an unknown yellow pigment could be chromatographically demonstrated which had IAA-photolyzing activity. Furthermore it should be mentioned that the reports on phototropic reactions in red light have been supplemented by Mohr (51) who observed responses of fern chloronema at wavelengths between 6000 and 7000 A. Further research is unquestionably needed before these findings

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which point to the participation of additional pigments in phototropic reactions can be fully evaluated.

Before turning to the mechanism which causes the differential growth of the anterior and posterior sides after unilateral irradiation and also the difference between the high phototropic sensitivity of the coleoptile tip and the low response of the subapical region, it seems desirable to describe several points more precisely. According to Cholodny (21) and Went (66) the differential growth of the opposite sides of bending coleoptiles and the dominating rôle of the tip is caused by a lateral migration of auxin, which is postulated to occur in an electrical field resulting from light absorption by the carotenoids (67). Changes in auxin metabolism are regarded as unimportant and are not considered in this theory. If this concept is accepted, one has to assume that phototropic responses and the light-growth reactions are different phenomena, based on different mechanisms (67). In contrast, the filter theory emphasizes the importance of light-dependent changes in auxin destruction and also in synthesis (60), which may both be mediated by flavins (26). Since these pigments are rather uniformly distributed throughout the entire length of the coleoptile, strong differences in the extent of the photochemical reactions and strong differences in growth of the illuminated and dark side can only be expected in the region of the tip where the screening effect of the carotenoids causes a steeper light gradient than in the basal parts, which are carotene-free and very transparent. In this manner, not only the high sensitivity of the coleoptile tip becomes understandable, but it is also possible to explain phototropic and light-growth reactions by the same photochemical processes.

Among the various investigations which have been elicited by the problematical situation described, a paper by Bünning *et al.* (15) should be considered first. They demonstrated that it is possible to raise considerably the low phototropic sensitivity of decapitated coleoptiles by artificial internal filters, and that the extent of the phototropic response of these objects is clearly dependent on the difference in the light intensity at the two sides of the coleoptile. Thus, after unilateral irradiation with weak daylight for two hours or more, transparent coleoptile stumps without the primary leaf developed only feeble curvatures. However, when their absorption was increased by retaining the leaf in the cavity or replacing it by a strongly absorbing solution of nigrosin, they reacted more strongly. In the presence of the primary leaf the bending was almost twice as large, and screening with nigrosin was sufficient to produce a reaction three times that of the stumps without primary leaves. This latter finding is therefore especially remarkable, for here a reaction capacity is attained which corresponds nearly to that of intact coleoptiles.

Obviously this is a model case which in optical aspects conforms precisely to the mechanism of the filter theory. The curvatures of the stumps can only be caused by asymmetrical light-growth reactions produced by flavin-mediated auxin destruction. The high efficiency of the internal filters

indicates moreover that the dominance of the tip region might actually be due to the "shadow effect" of the carotenoids. A problematical point in this paper is the authors' suggestion that a complete and satisfactory explanation of the phototropic responses in intact coleoptiles is also given by this mechanism.

Using an entirely different approach Henderson & Peterson (38) arrive at the same conclusion, namely, auxin destruction is the only essential factor in phototropism. They observed changes in the phototropic response of *Avena* coleoptiles after spraying with high concentrations of 2,4-D. In white light (approximately 2,000,000 mcs.) the normally occurring positive curvatures were totally prevented by high 2,4-D concentrations (1 gm./l.), while lower concentrations caused noticeable inhibitions. With the highest concentration of 2,4-D even negative curvatures induced by blue light could be reversed. In explaining these results the authors take for granted that high 2,4-D concentrations do not inhibit the growth of intact coleoptiles, and infer consequently, a specific effect of the synthetic auxin on the mechanism of phototropism. Noting that in previous investigations (37) 2,4-D inhibited IAA destruction in *Avena* coleoptiles, they assume that phototropism is solely the result of auxin destruction. Perhaps this method of approach is somewhat oversimplified and neglects too summarily the fact that after phototropic stimulation of coleoptiles destruction as well as synthesis of auxin has been observed (54).

A different view is held by Briggs, Tocher & Wilson (10). In order to find out whether light-induced changes in auxin metabolism or lateral auxin translocation are the cause of phototropic reactions they irradiated corn coleoptiles with white light (125,000 mcs.) and compared the yield of auxin diffusing from intact tips with others, partially split at the base or totally bisected by means of a cover slip. Though the light dosage was sufficient to induce strong curvatures in intact coleoptiles, they were unable to find any evidence in favor of auxin destruction. The total amount of auxin diffusing out of the tips within 3 hr. was the same with or without illumination. There was also no difference between the quantity of diffusible auxin on either side of irradiated tips which were separated by a cover glass. However, in illuminated tips divided only at the base three times as much auxin diffused from the dark as from the illuminated side.

If one, in regard to the preliminary nature of this work, neglects the unusually large variations of the phototropic reaction (angles between 29° and 84°) it is understandable that Briggs *et al.* ascribe the effect of light solely to the induction of a lateral auxin transport to the dark side. Yet we shall see later that this conclusion does not agree with other results on the same problem.

As to the failure to demonstrate an influence of light on auxin metabolism, one cannot eliminate the possibility that this may be due to the procedure selected. From all that we know about coleoptiles in this respect it would be surprising if this finding could be confirmed in more detailed ex-

periments with a different light dosage and a shorter diffusion time.

According to Brauner's (8) experimental data it is hardly doubtful that the rôle of auxin in phototropism is based upon a more complicated mechanism. In experiments with intact *Avena* coleoptiles which were cut at the base and whose light absorption was varied by removing or retaining the primary leaf and by injecting water or pigment solutions (india ink or crocin) into the cavity he observed effects corresponding to those of Bünning *et al.* (15) with decapitated coleoptiles. After unilateral irradiation of the whole coleoptile flank with incandescent light (60 mc. for 4 hr.) the extent of the developing curvatures increased with increasing absorption within the cavity. A loss in responsiveness, which was caused by covering the tips with small tin caps, also could be completely compensated for when the basal parts were provided with artificial filters. Moreover the normal difference in phototropic response when either the uppermost 5 mm. of the tip (higher carotene concentration) or the adjacent 5 mm. zone (lower carotene concentration) was illuminated almost completely disappeared after the absorption of the lower region had been increased by injection of india ink. It was also found that differences in the sensitivity of the tip in the transverse direction depend upon the carotene distribution. The narrow side containing a high concentration of these pigments was much more sensitive to phototropic stimuli than the broad side, which is almost free of carotenoids. Brauner feels that these observations are most reasonably understood if it is assumed that a lateral difference in auxin photolysis is caused by natural or artificial filters.

Brauner furthermore proved the participation of another factor in phototropic movements. Even when the differences in absorption of the uppermost tip and the adjacent 5 mm. zone were completely compensated, the tip region was more sensitive than the adjacent zone in the first phases of the reaction. He was also able to repeat the classic experiment of Boysen-Jensen (6) showing that a coleoptile whose tip was divided by a cover slip splinter along its shorter diameter bent about 60 per cent stronger upon illumination parallel to the incision than at right angles to it. In contrast to Boysen-Jensen (6) and Went (66), Brauner interprets this result not only in terms of lateral auxin migration, but states that the substantial bending after illumination perpendicular to the incision can only be due to differential auxin photolysis.

In discussing the implications of his findings, Brauner points out that two factors co-operate in the phototropic response of the coleoptile, a translocation of auxin and an asymmetrical auxin photolysis. The translocation is thought to be independent of a transverse light gradient and to occur in the transparent uppermost 200 μ region of the apex, while auxin photolysis should be active in the lower zone, where the carotenoids of the tip and of the primary leaf ensure the necessary light gradient. Although in a second paper (9) he leaves the question open as to whether a transverse field of force, electrical or possibly metabolic in nature, or an asymmetric decrease

in auxin permeability is the cause of the auxin deviation, there are a few points which need further consideration. As Nuernbergk (52) has shown, the uppermost part of the coleoptile (1 mm.) has peculiar optical properties, which might account for a transverse light gradient in this region despite the negligible amount of carotenoids present; upon illumination a distinct light concentration arises approximately 0.2 mm. behind the outermost cell layer of the "light" side. With this view there is no need to explain the high phototropic sensitivity of the apex by a process independent of a light gradient.

Another problematical point is the lateral auxin migration in a light-induced electrical field. In this regard several papers are of interest. Wiegand & Schrank (68) found that twice decapitated coleoptiles developed a transverse electrical polarity of 30 millivolts when a current of 20 μamp. was applied longitudinally to one side for 2 min. The side to which the current was applied always became negative and the polarity lasted for 50 to 70 min. As could be expected from earlier work (65) the stimulated coleoptiles bent as soon as IAA became available. Even though the induced polarity was independent of the current flow, different curvatures were obtained if the direction of the current was reversed. When the positive pole was inserted apically only curvatures toward the electro-negative side of the coleoptile (positive reaction) developed with IAA concentrations between 0.2–1.2 mg./l. However, opposite shunting resulted in weak positive curvatures at low IAA concentrations (0.2–0.8 mg./l.), while with higher IAA levels (1–1.2 mg./l.) these reversed to negative. In addition the loci of the curvature response remained always at the area of the initial stimulation, and maximum curvatures continued to develop when IAA was applied after the induced polarity had disappeared. Faced with these facts Wiegand & Schrank doubt whether conclusions (62, 65) can be maintained which support the assumption of a correlation between electrical and light-induced potential differences and lateral auxin transport. They believe that the electrical stimulation must affect a part of the growth mechanism which does not require auxin.

Further objections against light-induced lateral auxin transport have come from work with labeled IAA. In experiments with *Avena* coleoptiles Gordon & Eib (32) found that C^{14}-labeled IAA is transported in the same manner as the native auxin, i.e., strictly basipolar and with a velocity of 1 cm./hr. Equilateral or unilateral irradiation with a light dosage in the range of the first positive curvature did not alter the rate of transport or the distribution of this compound in the tip and proximal regions. Since unilateral irradiation with the same light dosage resulted in a higher concentration of endogenous auxin in the "dark" side, and there is evidence that the bulk of the free auxin of *Avena* coleoptiles is IAA (58, 61), it is very difficult to conceive that a simple transverse movement could be the cause of the observed auxin redistribution.

In connection with the results of Gordon & Eib, arguments of Bünning, Reisener & co-workers (16) against a light-induced migration of auxin also gain importance. In coleoptiles supplied with C^{14}-labeled IAA through an

apical cut which removed a part of the solid tip (200 μ) they were able to induce bendings by irradiation in the range of the second positive curvature. Yet when the coleoptiles were split 90 min. after the onset of the illumination, there was no measurable difference between the radioactivity of the two sides. Since the part of the apex was removed in which, according to Brauner (9), lateral auxin transport most likely occurs, these experiments are in the present stage not really convincing. Yet they could acquire considerable significance if it were shown in further experiments that the light dosage used did not affect the rate of isotope transport.

Although a definitive conclusion is not yet possible, it appears that light-induced changes in auxin metabolism and not a direct influence of light on auxin transport are the primary causes of phototropic movements. It can also be accepted as probable, that the light gradient in the organ itself determines the nature of these changes. A still open question is how under these prerequisites, the so-called "redistribution" of auxin, which depends apparently on processes induced by light in the uppermost part of the coleoptile tip, can be explained.

Until now only hypothetical considerations of this problem have been submitted. Galston (26, 27), on the basis of Oppenoorth's (54) results with ether-extractable auxin, suggested that differences in auxin production on the opposite sides of the apex cause the increased auxin content which has been observed, for instance, in the dark after stimulation in the range of the first positive curvature. Recently Meyer & Pohl (49) presented another hypothesis, in which auxin translocation is considered as a secondary effect originating from auxin destruction. They found products of IAA photolysis (indolealdehyde and indolecarboxyl acid) inhibited the growth of coleoptile segments only when applied apically, while submerged segments (no influence of auxin transport) were not inhibited. From these observations they deduce an inhibition of polar auxin transport by indolealdehyde and indolecarboxyl acid, and suggest that in phototropically stimulated coleoptiles the same compounds, by unilateral inhibition of transport, cause a deviation of the auxin flow. In this manner, with positive curvatures the auxin should be diverted to the dark side and with negative curvatures to the light side. In view of the results with C^{14}-labeled IAA (16, 32) this mechanism is somewhat improbable.

Fungi.—With the fungi, as in the higher plants, we are confronted with the question whether flavins or carotenoids are the receptors of phototropically active light. Substantial arguments in favor of the flavins as the actual photocatalyzers are the single-peaked action spectrum for the phototropic curvature of *Phycomyces* sporangiophores (20) which corresponds to the absorption of Rfl and other flavins, and the observations mentioned at the beginning of this review, that carotene-deficient sporangiophores react normally to light stimuli (43, 59). On the basis of carotene distribution in *Phycomyces* and the optical conditions in the sporangiophores (lens effect), which account for the mechanism of the bending by a transfer of a positive

light-growth reaction from the front to the rear (12), a filter action of the carotenoids can hardly be expected (59). An unsolved problem is how growth regulation takes place in fungi and how the presumed function of photocatalyzers can be fitted into this totally unknown system. There is some reason to suggest a flavin-sensitized photoinactivation of enzymes (26), IAA (7, 59), or "myco auxins" (1, 2) but this remains to be established.

Gettkandt (29), in studying the phototropic response of young hyphae of several phytopathogenic fungi, excluded the participation of carotenoids in these movements. In all species investigated only the uppermost tip regions (4 μ) were light sensitive and in contrast to the adjacent zones were evidently carotene-free. In experiments with broad spectrum filters, short-wave light (ultraviolet and blue) was effective, while wavelengths above 4500 to 4800 A proved ineffective. Since no account was taken of the relative light energies it seems logical to interpret these results as due to absorption by flavins, despite the fact that green light caused no response. Another interesting finding of Gettkandt concerns the mechanism of the reaction in the young mycelia. The phototropic response and likewise the light-growth reaction were negative; that is, the latter appeared as a growth inhibition. In addition a lens effect in the hyphae could be demonstrated; thus, the negative bending is understandable.

Further support in favor of flavins as photoreceptors in fungi has been derived from the field of developmental physiology. Page (55) was able to suppress the light-dependent formation of trophocysts in a *Pilobolus kleinii* strain by means of lyxoflavin, which is known as a competitive inhibitor of Rfl. The inhibition was almost completely reversed by the addition of Rfl. Diphenylamine, though inhibiting the synthesis of carotene in the fungus, had no effect on trophocyst formation.

Curry & Gruen (22) discovered, in contrast to the positive response in visible light, a negative phototropic reaction of *Phycomyces* sporangiophores in ultraviolet light caused by an acceleration of growth on the illuminated side. A rough action spectrum which was established for the positive (3000 to 5000 A) and the negative bending (2400 to 3000 A) showed a distinct minimum at 3000 A (zero response and reversal of phototropism) and a strong maximum near 2800 A, falling off and rising again at shorter wavelengths. The authors feel the lack of response at 3000 A and the course of the action spectrum in the short-wave range are best understood if positive and negative responses are considered to be different phenomena. Accordingly, the negative reaction is ascribed to the action of an unknown photoreceptor (peak absorption at 2800 A) and is thought to be independent of the optical properties of the sporangiophore (lens effect).

However, Carlile (19), who compared the action spectrum established by Curry & Gruen with the absorption curve of Rfl, found a remarkable conformity of both curves in ultraviolet and visible light; hence, he suggested that negative and positive curvatures are due to a single Rfl-mediated reaction. The reversal of both tropisms at about 3000 A and the negative response

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at shorter wavelengths is explained in optical terms. As Carlile points out the absorption coefficient of the sporangiophore at 3000 A reaches Castle's critical value. Thus the lens effect can not bring about greater photochemical activity on the far side. With still shorter wavelengths strong absorption by the protoplasm almost completely screens out the radiation and the result is a higher light intensity on the near side which produces the negative bending. Though this explanation appears plausible, it contains an ambiguous point. If the reversal of phototropism is explained in optical terms, then the phototropic action curve minimum about 3000 A must be ascribed to an equalization of the light intensity on both sides of the sporangiophore and not to the minimal absorption of flavins in this part of the spectrum. At present, therefore, the similarity between the absorption curve of Rfl and the phototropic action spectrum cannot be considered as a convincing proof for the participation of flavins in negative curvatures (3). A decision on this question should be possible when a determination of the action spectrum for the light-growth reaction of sporangiophores is achieved, for with equilateral illumination the absorption of the photoreceptor and not the lens effect determines the extent of the light response.

The suggestion that positive light-growth reactions and phototropism in *Phycomyces* might arise through partial photolysis of previous supra-optimal concentrations of IAA has been tested by Gruen (34) and Banbury (3). Gruen, who applied IAA and the auxin antagonist indoleisobutyric acid in small water droplets to one side of the sporangiophores, found no effect of these compounds on the bending which could be induced by water alone. Banbury tried to decide the matter in a different manner. He applied small patches of lanolin paste and sodium alginate gel containing IAA (10 mg./l. and 100 mg./l.) unilaterally to the growing zone of sporangiophores, which had been either previously subjected to intense illumination or, after IAA application, given a light stimulus from the opposite side. Likewise, he observed no change in the response which could be brought about in the controls with pure lanolin paste and alginate gel alone. Thus Banbury concludes that the participation of IAA in any light response of fungi is rather questionable. Gruen, however, goes much further and derives conclusions which are not firmly proved by his results. He postulates that water supply and not auxin is the limiting factor in the elongation of the sporangiophores. This may be true under the experimental conditions used, but in order to exclude any participation of IAA or "myco auxins" it must also be demonstrated that in water-saturated sporangiophores substances like IAA, indoleisobutyric acid, and griseofulvin do not influence light-induced movements. The best method to decide this matter would be the use of Buder's technique with submerged objects.

Phototaxis of Flagellates

The term phototaxis designates two different types of photic orientation in freely moving organisms, i.e. topo- and phobophototaxis (11, 36, 56).

Topotactic movement is related to the direction of the light, for in this case the algae move toward or away from the light source (positive and negative reaction). On the contrary, phobotaxis is independent of the light direction and evidently depends upon "shock reactions" induced by changes in light intensity which result in endogenously fixed alterations in the direction of the movement. In this way the organism can be constrained to stay within or to leave a light trap (positive and negative response). With unicellular flagellates there is some disagreement in the explanation for the mechanism of topotaxis. It has been considered either as consisting of a series of phobic reactions (11, 17, 39, 46, 57) and therefore termed "pseudo-topotaxis" (39, 46), in which the orientation is postulated to be dependent on a periodic shading of the photoreceptor (11, 17, 39, 46), or as a mechanism totally different from phobotaxis (4, 11, 35, 45). The orientation could then possibly be brought about by the illumination (positive response) or by the shading (negative response) of the photosensitive spot (35).

Conclusions as to the nature of the pigments active in phototaxis are based almost exclusively on investigations of the spectral sensitivity of the algae. The first reliable information on the phototactic action spectrum of unicellular algae was reported by Engelmann (24). Using a micro spectrum, he observed the maximum accumulation of *Euglena* at about 4800 A. With *Phacus*, *Trachelomonas* and various *Euglena* species Mast (46) and Oltmanns (53) also found this region most active. From the experimental procedure used it is possible that Engelmann and Oltmanns investigated the phobic response, whereas Mast with his method determined with certainty the action spectrum of topotaxis. Later the maximum in the blue-green light has been repeatedly verified for unicellular and colonial forms of flagellates (44, 45, 48). The most exact methods (spectral lines) were employed by Luntz (45) who with various green forms (*Chlamydomonas*, *Eudorina*, *Volvox*) established maximal phototactic activity around 4900 A, while wavelengths above 6500 A had no effect.[4] On the other hand a colorless *Chilomonas* without stigma, which he likewise examined, reacted most strongly at 3660 A. Luntz (45) and Pringsheim (57) explained the strong effect of blue-green light through the absorption of these wavelengths by the carotenoids of the stigma, whereas the effect of long-wave ultraviolet radiation was ascribed by Luntz to the sensitivity of the protoplasm. Today neither of these assumptions is tenable, for effective absorption of protoplasm can be counted on first at wavelengths under 3000 A, and it has become evident with *Euglena* that the absorption curve of the pigments in the stigma and the action spectrum of phototaxis are different (33).

A new approach to the identification of the photoreceptive pigments in flagellates was achieved in recent experiments. At present, however, it is still

[4] Metzner's report (48) on phobic responses of *Ceratium* in red light has attracted little attention up to now. Since Wolken & Shin (69) also recently observed phototactic reactions of a green *Euglena* in red light, it seems advisable to investigate thoroughly this region of the spectrum in the future.

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uncertain whether only one or possibly several pigments, which are different in nature, are involved in the phototaxis of flagellates. The nature of the pigment or pigments remains unknown, but apparently it is neither a flavin (33) nor any of the major pigments in algae (35).

For negative phobotaxis of *Euglena*, Bünning & co-workers (17, 18, 33) found in visible light a maximum at 4250 A and two minor maxima at 4500 and 4800 A. Wavelengths above 5500 A proved to be inactive. The remarkable thing about these results is the fact that the course of the action spectrum was in general the same for both a green form with and a colorless form without stigma (Fig. 1). Investigations of the positive topic response of the same strains likewise led to interesting results. In contrast to the phobotaxis, the maximum for the topic movement was found at 4950 A; but this applies only to the action spectrum of the green form, whereas the colorless strain responded strongest to violet light with a maximum at 4100 A (Fig. 2).

Despite the different action spectra, Bünning *et al.* attribute the absorption of phototactically active light to the same pigment. A prerequisite for this thesis is the assumption that illumination of the photoreceptor induces only phobic responses. Thus, the action spectrum of phobotaxis (in which no periodic shading is necessary) corresponds directly to the absorption of the actual photoreceptor with a maximum around 4250 A. Topotaxis is a different matter, to be considered as "pseudo-topotaxis." For this type of movement, alterations in light intensity which are strongest when the shading body and not the photoreceptor absorbs maximally are decisive. In green forms of *Euglena* the shading is accomplished by the stigma [maximal absorption at 4800 A (33)] which is in compliance with the maximal response in blue-green light. In colorless strains without stigma this function is apparently performed by other parts of the cell absorbing blue-violet light, hence the reaction maximum at 4100 A.

Another concept is advocated by Halldal (35) who found no differences between the action spectra for positive and negative topotaxis. The shape of the curves for unicellular *Volvocales* closely corresponded to the previous findings with *Euglena* (Fig. 3). An exception was found in Dinophyceae, for two species reacted maximally at 4750 A whereas with a third species (*Prorocentrum micans*) a maximum was observed at 5700 A. In analyzing these action spectra Halldal starts with the assumption that phobic and topic responses are completely different phenomena, and postulates that positive topotaxis occurs with illumination and negative with shading of the photoreceptor. If, however, as Halldal further argues, under these conditions the action spectra are identical, then the shading effect of the stigma or the plastids has no substantial influence on the shape of the action spectra. Hence these can only be determined by the absorption of the photoreceptor. Since at least the action spectrum of one species (*Prorocentrum micans*) was definitely different from that of the other species investigated, Halldal maintains that at least two different pigments are involved in the phototaxis of these algae.

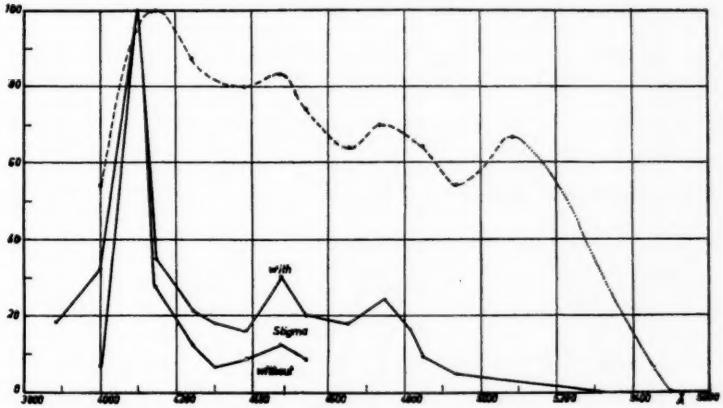

FIG. 1. Action spectrum for negative phobo-phototaxis of *Euglena gracilis*.
– – – – – = green form with stigma.
——— = colorless forms with and without stigmas.
Abscissa = wavelength in A.
Ordinate = phototactic sensitivity in relative units.
[From Bünning and Schneiderhöhn (1956) and Gössel (1957).]

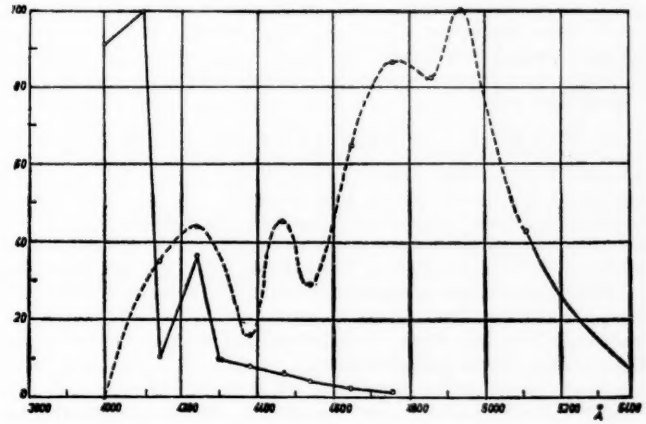

FIG. 2. Action spectrum for positive topo-phototaxis of *Euglena gracilis*.
– – – – – = green form with stigma,
——— = colorless form without stigma.
Abscissa = wavelength in A.
Ordinate = phototactic sensitivity in relative units.
[From Bünning and Schneiderhöhn (1956) and Gössel (1957).]

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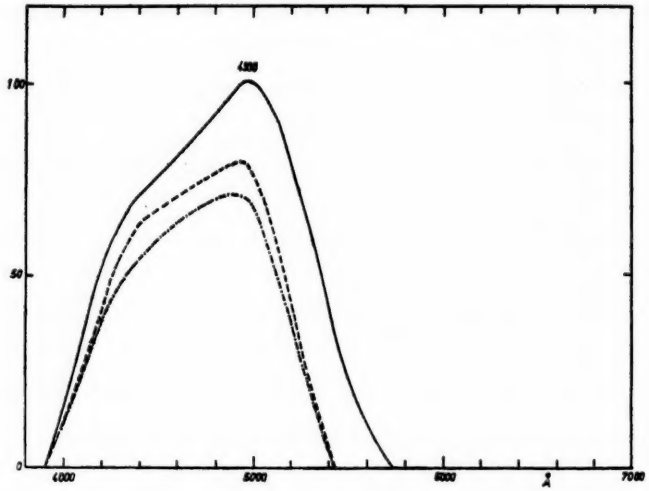

FIG. 3. Action spectrum of positive and negative phototaxis in unicellular Volvocales.

– – – – – – = *Dunaliella.*
– · – · – · – = *Stephanoptera.*
———— = *Platymonas.*
Abscissa = wavelength in A.
Ordinate = phototactic sensitivity in relative units.
[From Halldal, 1958].

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